Exploring marine sponges as a source of novel chemical entities for drug development

A thesis submitted for the degree of

Philosophiae Doctor (PhD)

by

Christopher Martin Hatton, MPharm

Cardiff School of Pharmacy and Pharmaceutical Science

Cardiff University

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"They may look like plants but sponges are one of the simplest of all living animals, yet in their own way they are amazing"

-Sir David Attenborough

Attenborough's Ark, Natural World Special, BBC, 5/11/2012

Abstract

Antibacterial resistant infections are one of the most challenging problems affecting healthcare and have developed through the overuse of antibiotics and a shortage of new treatments progressing to market. Natural products are the initial source of most antibiotics currently available and marine sponges are a known resource of novel antibacterial compounds; although well-studied marine sponges found in UK waters have been scarcely explored.

An examination of the chemical research on sponges identified previously unstudied species for collection in both Greece and Wales. Sequential solvent gradient extraction was optimised, to best exploit the material collected, providing three crude extracts for each sponge collected. A significant difference was observed between the chemical composition of sponges collected from Greece and Wales.

An efficient antimicrobial assay was developed to screen each extract against clinically relevant organisms; allowing the direct identification of activity on an eluted thin layer chromatography plate. This overlay data was used for detailed chemical analysis using high performance counter current chromatography, with some separated fractions displaying greater activity towards the bacterium methicillin resistant *Staphylococcus aureus* (MRSA) than vancomycin. The parent masses of compounds responsible for activity were identified by directly coupling the overlay assay data to mass spectrometry, identifying multiple novel parent masses. Dereplication of samples was completed using the database MarinLit and the construction of a molecular network to compare fragmentation patterns in mass spectra.

Bacterial cultivation from Welsh sponge samples isolated 18 antibacterial strains, which were identified using 16S rRNA analysis. Four of these strains were previously uncultured. Chemical analysis was also completed, on two unstudied strains, identifying further active novel parent masses, with no parent mass crossover to the host sponge.

Overall, this investigation concluded that marine sponges are excellent source of novel antibacterial compounds, which can display activity against clinically relevant bacteria equivalent to current treatments.

Table of Contents

Abbreviations

ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
CCC	Counter current chromatography
CFU	Colony forming units
DCM	Dichloromethane
DEFRA	Department for Environment, Food and Rural affairs
diH_2O	Deionised water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleoside triphosphate
DoH	Department of Health
EtOAc	Ethyl Acetate
FDA	Food and Drug Administration
g	Gram
h	Hour
HAI	Hospital acquired infection
HPCCC	High-performance counter current chromatography
HPLC	High-performance liquid chromatography
ICU	Intensive care unit
INT	Iodonitrotetrazolium chloride
L	litre
LC	Liquid chromatography
m	Metres
min	Minute
M	Molar
MA	Marine agar
MB	Marine broth
MBC	Minimum bactericidal concentration
MeOH	Methanol
MHA	Müller-Hinton agar
MHB	Müller-Hinton broth
MIC	Minimum inhibitory concentration
mg	Milligram

mL	Millilitre
MNPs	Marine natural products
MRSA	Methicillin resistant *Staphylococcus aureus*
MS	Mass spectrometry
MSSA	Methicillin sensitive *Staphylococcus aureus*
m/z	mass-charge ratio
MW	molecular weight
n	Number of replicates
NB	Nutrient Broth
NCIMB	National Collection of Industrial, Food and Marine Bacteria
NCTC	National Collection of Type Cultures
nm	nanometre
NMR	Nuclear magnetic resonance
OD	Optical density
OTU	Operational Taxonomic Unit
p	Value of significance
PANDAseq	Paired-end assembler for illumina sequences
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEAR	Potential-based entropy adaptive routing
r	r-value (Pearson product-moment correlation coefficient)
r^2	Coefficient of determination
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
SCUBA	Self contained underwater breathing apparatus
SD	Standard deviation
SE	Standard error of the mean
t	t-test value
TLC	Thin layer chromatography
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UK	United Kingdom
USA	United States of America
UV	Ultraviolet

v/v	Volume per volume
w/v	Weight per volume
°C	Degrees Celsius
η^2	eta squared ratio (ratio of variance

Chapter 1
Introduction

1 Introduction

1.1 Marine life

The ocean covers over 70% of the earth's surface and was the home of the first living animals (Ausubel *et al*. 2010). Marine organisms have evolved to thrive in the extreme living conditions encountered in marine environments including high salinity, low temperatures and low availability of nutrients (Newman and Cragg 2004; Hill and Fenical 2010; Samuel *et al*. 2011; Lawrence 2015). The census of marine life project estimated that there are at least one million marine species, only one quarter of which are known to science; and tens of millions of marine microbial species, most of which have not been discovered or identified (Ausubel *et al*. 2010).

1.1.1 Classifications of animals and marine life

Living organisms, found in a marine environment, can be classified using the following three methods-

1. Marine organisms can be divided into three categories depending on their mode of life (Wood 2007; Castro and Huber 2012):
    - **Nektonic** organisms are able to move and swim freely *e.g.* fish, marine mammals and squid.
    - **Benthic** organisms are rooted to the sea bed including creeping species like crabs and snails, sessile animals such as sponges and corals and burrowing animals like clams and worms
    - **Planktonic** organisms are microscopic with no power of movement and usually drift or float with the current *e.g.* plankton
2. Marine multi-cellular organisms are also commonly differentiated by whether or not they have a backbone (Wood 2007; Castro and Huber 2012):
    - **Vertebrate** have a backbone *e.g.* fish and dolphin.
    - **Invertebrate** are without backbone *e.g.* Sponges, crabs etc.
3. Marine organisms can be classed into one of the following six kingdoms (Wood 2007; Castro and Huber 2012).
    1. **Animals** are large and multi-cellular. They are heterotrophic and live by feeding on other organisms. Marine animals include fish, sponges, crustaceans and jellyfish.

2. **Bacteria** are small single cell organisms that reproduce by binary fission. They are found throughout the marine environment and perform a critical role in marine ecosystems by recycling organic material are thereby freeing up nutrients for other organisms.
3. **Protozoans** (a dated term) are larger single celled organisms. They are able to feed in either a heterotrophic or autotrophic manner and are well represented in the marine environment. The classic example of a protozoan is an amoeba (Denyer *et al*. 2011).
4. **Chromists** vary from very small organisms like diatoms to large seaweeds. The majority of chromists are able to feed through photosynthesis, utilizing a different kind of chlorophyll to plants, and it is due to such differences that scientists do not classify them as plants.
5. **Plants** are multicellular and feed in an autotrophic manner using photosynthesis to produce their food from sunlight. Few types of plants thrive in the marine environment with many being classified as chromists. An example of a marine plant is sea-grass; seaweed is now classed as a chromist.
6. **Fungi** live by metabolising organic material and are unable to create food of their own (Denyer *et al*. 2011). Fungi are generally not well represented in the marine environment.

Some very simple animals are often difficult to characterise as animals and are commonly mistaken for plants or other life forms. This is especially hard with basic life forms, which are sessile such as marine sponges. Animals normally conform to the following characteristics (Castro and Huber 2012):

- **They are heterotrophic.** They obtain energy by consuming other organisms. Marine animals have developed various novel ways to achieve this. The whale shark for example is the largest of all fish yet it consumes tiny plankton by mobile filter feeding. It sustains itself by travelling the world following plankton blooms with the varying seasons. Sponges opt for stationary filter feeding, which proves to be extremely efficient, and wait for the food to come to them (Sprung 2001).
- **They are able to move.** The majority of animals are able to move but there are a number of examples where this may difficult to spot *e.g.* Barnacles. Sponges however are totally stationary and do not conform to this part of the definition.

- **Their body forms are an arrangement of multiple cells.** In the majority of animals these multiple cells form tissues, which perform specific functions. This separates animals from other forms of life such as bacteria and prokaryotes, which are made up of just a single cell.
- **Reproduce Sexually.** Most animals reproduce sexually through the process of sperm combining with an egg. Some animals such as sea stars are able to reproduce asexually by binary fission.

Marine animals can therefore be classed using all three of the methods outlined above. For example: the great white shark (*Carcharodon carcharias*) would be classed as a nektonic animal with vertebrate (made up of cartilage not bone). A marine sponge would be classed as a benthic invertebrate animal as it lives on the bottom of the ocean and has no backbone.

1.2 Marine sponges (phylum Porifera)

Sponges make up a significant proportion of the benthic community (Taylor *et al*. 2007) but they are amongst the oldest and most basic of all multicellular animals (Yin *et al*. 2015). Sponges possess very few characteristics that we usually identify with animals. They are rooted to the sea bed, have no movement and they derive their oxygen and nutrients from passing currents of sea water running through their body (Hooper *et al*. 2002). Sponges are known to grow in both fresh and salt water but are primarily found dwelling on the bottom of oceans in nutrient rich environments that have strong currents (Wood 2007). They are sessile filter feeders and they retrieve their food from the surrounding water in a very efficient manner despite their simplicity (Taylor *et al*. 2007).

Their scientific classification as phylum Porifera literally means *pore bearing* and is derived from the surface of the sponge, which has a large number of holes and appears 'sponge-like'. This porous sponge-like surface is held together by a hard skeleton which is composed of spicules (calcium carbonate, spongin (a collagen protein that forms flexible skeleton) or siliceous material) (Wood 2007). Most of these 'pores' are microscopic and are not visible with the naked eye. These small holes are in current, where water is drawn in (Figure 1-1). Water is pulled into a system of canals and channels by a layer of tiny whip-like cells with flagella, which are constantly moving, similar to those found in the human airway. Oxygen and food is extracted as the water passes through the sponge body and is expelled through the bigger pores, which are usually visible to the naked eye. The large ex-current pores (oscula) are usually located on the extremities of the sponge (Figure 1-1) to ensure the same

water does not recirculate through the body. Sponges cells grow differently depending on the type of species, with cells growing at the end of branching species, at the edge of encrusting species and across the surface of rounded forms (Wood 2007). Sponges are structurally basic, have no cellular tissues but do have cellular level organization meaning specialist cells perform distinct tasks (Hooper *et al*. 2002).

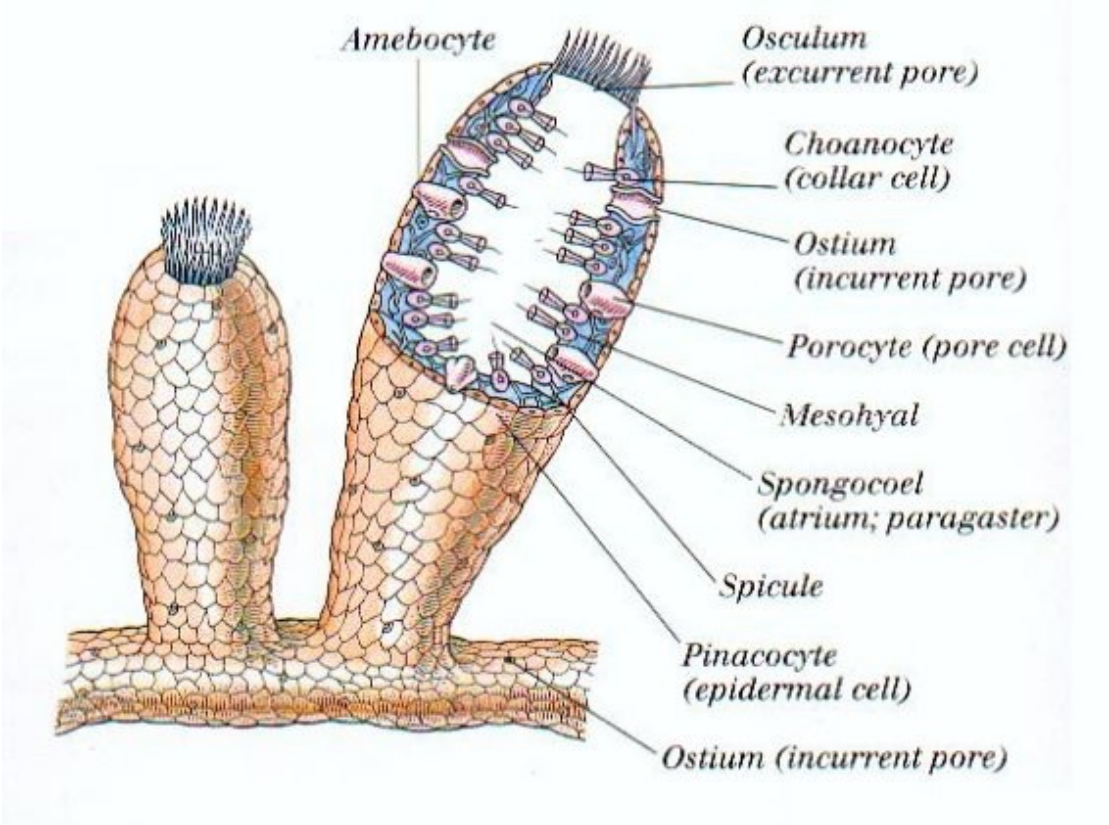
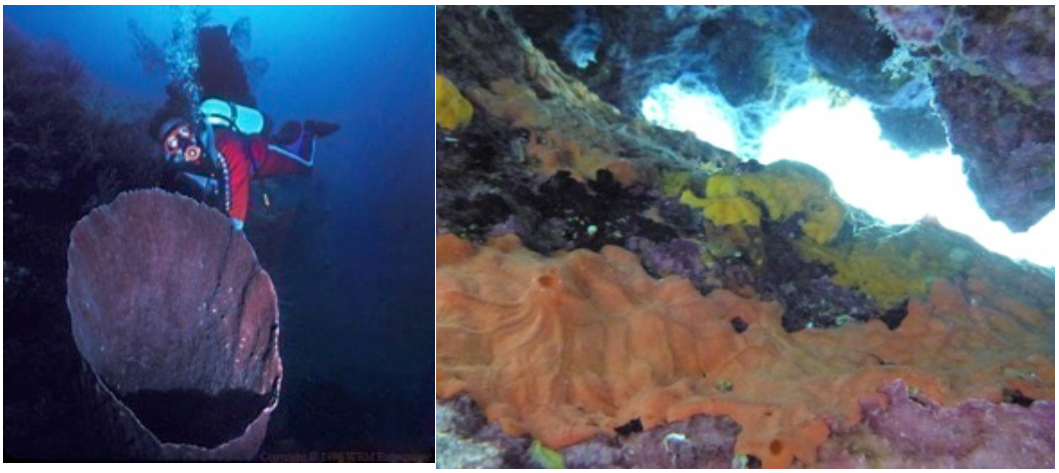


Figure 1-1 Cross sectional diagram of a typical marine sponge taken from (Frazer 2011)

1.2.1 Types of sponges

Sponges come in many different shapes and form, from the giant barrel sponge (A, Figure 1-2) to the barely visible thin sheet and encrusting sponges (B, Figure 1-2).



(A) **(B)**

Figure 1-2 Images of (A) giant barrel sponge and (B) encrusting sponge species.

There are over 6000 taxonomically different species of sponge that can be found world-wide (Grozdanov and Hentschel 2007). They are found to flourish in a range of different conditions from salt to freshwater and from tropical and temperate climates to polar-regions. Of these, 350 species can be found within the United Kingdom and its surrounding seas (Goodwin and Picton 2011). They can be extremely difficult to identify in their natural habitat and often require microscopic analyses by an expert. The arrangement of skeletal spicules, that are found throughout the sponge and give them their form, is often used in microscopic species identification (Hooper *et al*. 2002).

The most common forms of sponge are listed below (Wood 2007; Sprung 2001; Hooper *et al*. 2002):

- **Branching** – the body is branched, forming 'tree-like' or 'stag-horn' forms, which can be in one plane like a fan.
- **Boring** – where the majority of the sponge has dug itself into a rock such as limestone. In a similar manner to an iceberg in water, where only a portion of the sponge is visible.
- **Thin sheets** – very thin encrusting sheets that cover rock surfaces, few features visible to the naked eye, could easily be mistaken for algae (Figure 1-2).
- **Cushions** – thicker encrusting sheets up to 10 mm thick covering rock but containing visual canals and holes (Figure 1-2).
- **Massive** – rounder form that sticks out significantly from the surface they are attached to.

- **Cylindrical** - narrow base with a wider and taller cup or cylinder like body. Including the giant barrel sponge (Figure 1-2).
- **Lamellate** – the growth is predominantly vertical and normally in one plane producing a fan like or plate like form.

1.2.2 Classes of sponge

The phylum Porifera is subdivided into three groups based upon the structure of the skeletal elements that hold the sponge together (Sprung 2001; Wood 2007; Hooper *et al*. 2002).

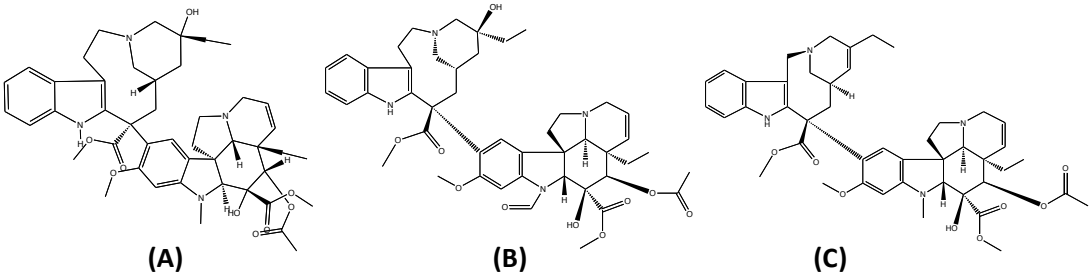
1. **Demospongiae** (desmosponges). Represent the most abundant and most varied class of sponge and encompass over 90% of known sponges. They show great diversity in appearance and size, varying from a few millimetres to over 2 m in width. They are renowned for the vast array of colours in which they appear. Evolution has enabled them to grow in a variety of locations from shallow tropical waters to the dark cold abyss. They are also the only sponge family found in freshwater. Structural rigidity is achieved through a skeleton which consists of either siliceous spicules (containing one to four rays), course sponging fibres, or both (Hooper *et al*. 2002).
2. **Hexactinellida**. More commonly referred to as glass sponges and of which approximately 500 different species have been identified in the wild. They are commonly found in Antarctic waters, where they represent the most obvious form of benthic life in the freezing waters. Their appearance is always upright and often symmetrical, shaped like a vase or funnel and can form structures as large as 1 m^3. Structural rigidity is achieved through a hexactinelid skeleton and they can be distinguished by their six rayed siliceous spicules, which form a framework. This framework causes a structural rigidity that is not found in any other sponges (Hooper *et al*. 2002).
3. **Calcarea**. Named after their framework because they contain spicules made of calcium carbonate. 100 Species have been identified and they are usually found in marine water no deeper than 1000 m although one species has been found at a depth of 4000 m. They tend to be small (<10 cm) and they demonstrate simple shapes such as vase, pear or a cylindrical shape (Hooper *et al*. 2002)

1.3 The medicinal use of natural products

1.3.1 Current and historical place of natural products in drug therapy

Humans have always relied upon substances from the natural world as a tool to survive, by not only using natural products as a source of sustenance but also as medicines. Historically there is evidence of the Egyptians using medicines as early as 2900 BCE (Cragg *et al*. 2009). Another good historical example can be found from the Mesopotamia era (~2600 BCE) where records document more than 1000 plant-derived medicines, many of which are still used today to treat coughs, colds and infections. More clear evidence is seen in the Ebers Papyrus, which was produced in 1500 BCE and records more than 700 drugs (Cragg *et al*. 2009).

Natural products continue to play a huge part in healthcare all over the world including 'modern medicine'. The World Health Organization estimates that around two thirds of the world population rely predominantly on traditional medicines derived from natural products. Natural products are not just used as herbal medicines, with estimates suggesting over 50% of drugs prescribed in the USA are of natural origin (Jia 2003). 'Modern medicine' still relies heavily on historical use of plants and several current anticancer therapies were developed from their use as traditional medicines. The vinca alkaloids (Figure 1-3), which are used today for the treatment of acute leukaemia were originally isolated from a flowering plant, the Madagascan Periwinkle (Cragg *et al*. 2009).



(A) **(B)** **(C)**

Figure 1-3 Structures of vinca alkaloids. (A) (+)-Vinblastine; Formula: $C_{46}H_{58}N_4O_9$; Mass: 810.42, (B) Vincristine; Formula: $C_{46}H_{56}N_4O_{10}$; Mass 824.40, (C) Vinorelbine, Formula: $C_{45}H_{54}N_4O_8$; Mass 778.39.

1.3.2 Natural products as source of novel metabolites for drug development

Historically, natural products have proved an inexhaustible source of structurally diverse compounds in the fields of medicine, pharmacy and biology (Gordaliza 2007). From the 1960s, novel metabolites derived from plants and microbes have proved to be useful

anticancer drugs (Kinghorn *et al*. 2009). Marine fauna and flora as well as surface-dwelling microorganisms have been tested for their anticancer properties because of the potent agents that have been found before.

Plants and marine invertebrates employ natural products for may different reasons including chemical defence against predation. For the marine sponge this is essential, as it has no other weaponry to keep predators away or suppress the development of neighbouring species protecting their territory (Proksch 1994; Luter and Duckworth 2010). Microorganisms similarly manufacture toxins with the ability to kill sensitive strains of competitive species (Cragg *et al*. 2009). The metabolites these simple organisms produce, as a defensive mechanism, could potentially provide leads as novel anticancer and anti-microbial agents.

A lead compound is a novel biologically active structure, which has potential as a therapeutic. Natural products are commonly used by the pharmaceutical industry as lead structures which are later rationally modified to give compounds with greater selectivity, pharmacological activity and improved toxicological profiles (Gordaliza 2007) (Figure 1-4).

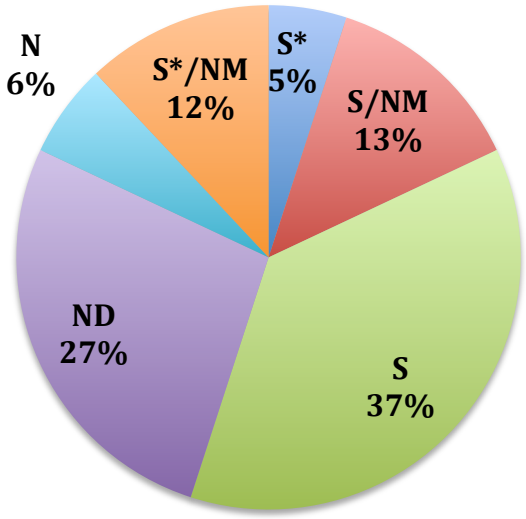


Figure 1-4 Small molecule new chemical entities 01/1981-10/2008 by Source (*n* = 1024). Drugs were classified as N (an unmodified natural product); ND (a modified natural product); S (a synthetic compound with no natural product conception); S/NM (a synthetic compound showing competitive inhibition of the natural product substrate); S* (a synthetic compound with a natural product pharmacophore); and S*/NM (a synthetic compound with a natural product pharmacophore showing competitive inhibition of the natural product substrate) adapted from Cragg *et al*. (2009).

From the 98 currently licensed antibacterial drugs 74 originate from structures derived from nature (Zhang and Demain 2010) with 9 out of 12 of the major antibacterial classes originating from nature (Zhang and Demain 2010). Figure 1-4 shows that only 37% of chemical entities 01/1981-10/2008 were totally synthetic, which means a 63% of novel chemical entities originate from natural products.

Recent successful pharmaceutical application of several natural products (Newman and Cragg 2007), has led to an increase in interest, amongst biologist and chemists, in these methods of isolating active compounds and consequently the supply of these natural products may be enhanced (Ling *et al*. 2015). However many scientists have no intention of using directly extracted but are looking for inspiration for the production of new drugs. Isolated natural products can be used to determine preliminary structure activity relationships and potential mechanisms of action of known active compounds (Kinghorn *et al*. 2009; Coseri 2009). Once a compound is isolated and found to be active its structure can be modified for potential improvements. Chemists can then produce alternate but similar structures which they can manufacture or modify (semi-synthetic compounds) and then test their efficacy in an attempt to find the perfect compound with greater activity and fewer side effects (Gordaliza 2007).

1.3.3 Marine life as a source of natural products

The marine habitat provides one of the harshest environments on the planet so organisms need to find a competitive edge, which leads to the production of diverse molecules as a manner of chemical defence. As of 2015, 26,490 novel natural products (*MarinLit* compound search, 2015) have been isolated from marine origins. Approximately one third of those discovered to date originate from marine sponges, making them one of the richest sources of marine novel natural products (*MarinLit* compound search, search taxonomically limited by "Porifera', 2015). This figure is made all the more startling by the fact that research in marine habitats has been virtually unexplored in comparison to terrestrial habitats (Hughes and Fenical 2010). It is estimated that an increased effort in the field of drug discovery from marine microbe sources could potentially yield 100 times the number of novel compounds for the pharmaceutical industry (Hill and Fenical 2010).

Marine sources are recognized for their incredible biodiversity making them ideal candidates for discovering novel cytotoxic and anti-infective agents. This is highlighted by the relatively high number of marine natural products (MNPs) in pre-clinical and clinical development

(Schwartsmann *et al*. 2003). Didemnin B (Figure 1-5) was the first marine peptide that entered in human clinical trials in the USA for the treatment of cancer (Singh *et al*. 2008). While didemnin B is no longer in clinic trials due to anaphylactic reactions, the structurally related aplidin remains in trials.

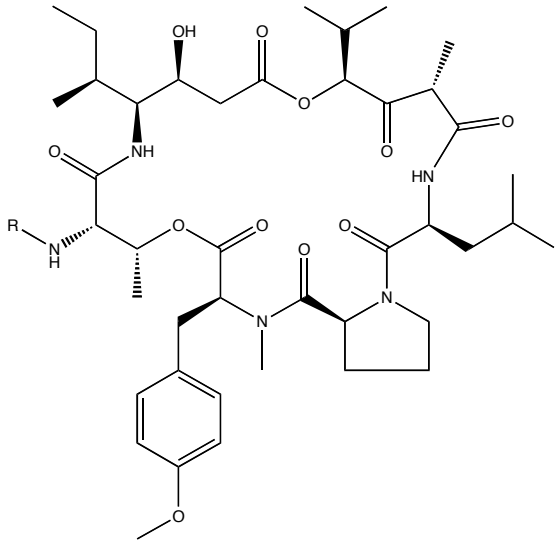


Figure 1-5 Structure of didemnin's. R = *N*-Me-L-Leu (didemnin A). R = Lac-Pro-*N*-Me-L-Leu (didemnin B). R = Lac-*N*-Me-L-Leu (didemnin C).

MNPs are known for their diversity in structural class and vary from basic alkaloids, to simple linear peptides, to complex macrocyclic polyethers. Advances in technology for the isolation and characterization of products and development of high throughput screening, have made it much easier and have significantly increased the rate of discovery of new compounds with medical potential (Singh *et al*. 2008). Marine environments provide an almost limitless supply of structurally diverse natural products. With the oceans potential largely untapped, many new compounds are waiting to be discovered and a rational approach is needed for this (Dinan 2005).

1.4 Marine sponges as a source of novel metabolites for drug development

Considered one of the most basic of animals, marine sponges are already a proven source of active MNPs such as anti-infective agents (Ankisetty and Slattery 2012) and cytotoxic drugs (Youssef *et al*. 2013). Marine sponges were one of the first marine organisms to be investigated for their interesting compounds (Fattorusso *et al*. 1970; Forenza *et al*. 1971) and MNPs have now established themselves as a primary target for novel leads due to the vast

biodiversity found in the world's seas (Blunt *et al*. 2014; Hill and Fenical 2010; Laport *et al*. 2009). Nucleosides, which were gathered from a Caribbean sponge, were the original inspiration for the development of antiviral, modified nucleosides and the cytotoxic agent cytarabine, which is still used today to treat lymphomas and acute leukaemia (Schwartsmann *et al*. 2003; Molinski *et al*. 2009; Roboz 2012). When compared to other marine invertebrates the number of novel compounds isolated from marine sponges is similar to that of all the other invertebrates combined (Figure 1-6).

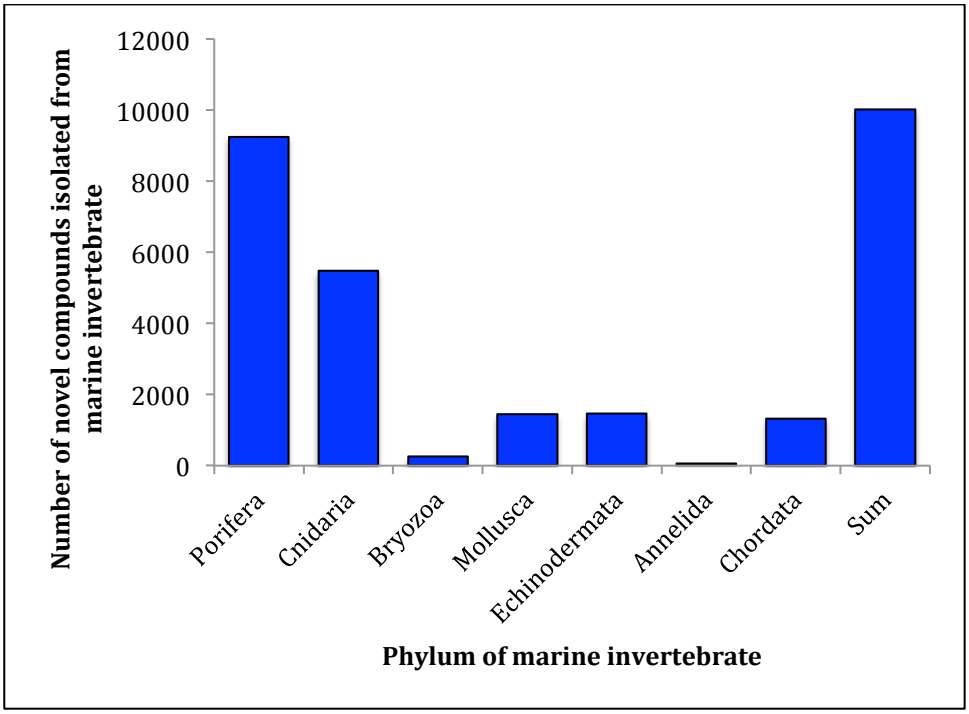


Figure 1-6 The number of novel compounds isolated from marine invertebrate up to 2015. Calculated by applying taxonomic limitations in *MarinLit* and recording the number of compounds reported. The sum of all other invertebrates apart from marine sponges is also displayed.

Sponges are interesting for two main and often related reasons: they form close and potentially symbiotic relationships with a variety of microorganisms and they are a rich source of biologically active secondary metabolites (Taylor *et al.* 2007). Secondary metabolites are compounds produced by an organism that have no direct involvement in growth, development or reproduction (Fraenkel, 1959). The most commonly isolated MNPs from marine sponges can be categorized into the following four groups (Fattorusso *et al*. 2012):

1. Peptides
2. Polyketides (*e.g.* macrolides and eribulin)
3. Alkaloids (*e.g.* Manzamine, bromopyrrole and bromotyrosine)

4. Terpenes (*e.g.* sesterterpenes and triterpenes)

1.4.1 Peptides

Marine sponge derived peptides, both antimicrobial and non-ribosomal, already represent a significant area of MNP research and offer unique structures in comparison to other sources. They come in both cyclic and linear forms with unusual amino acid groups not found from microbial and terrestrial sources (Fattorusso *et al. 2012)*. Marine derived peptides have been shown to display a range of biological activities including antifungal, cytotoxic anti-inflammatory and antibacterial. The antibacterial discodermin peptides (Matsunaga *et al.* 1985) were originally isolated from the marine sponge *Discodermia kiiensis* and have since been isolated form the genera of *Ircinia* and *Halichondria*. In general they contain a mixture of 13-14 common and rare amino acids and form a macrocyclic ring through the lactonization of threonine. The antifungal and cytotoxic halicylindramides represent similar compounds to the discodermins but with alterations in amino acid residues.

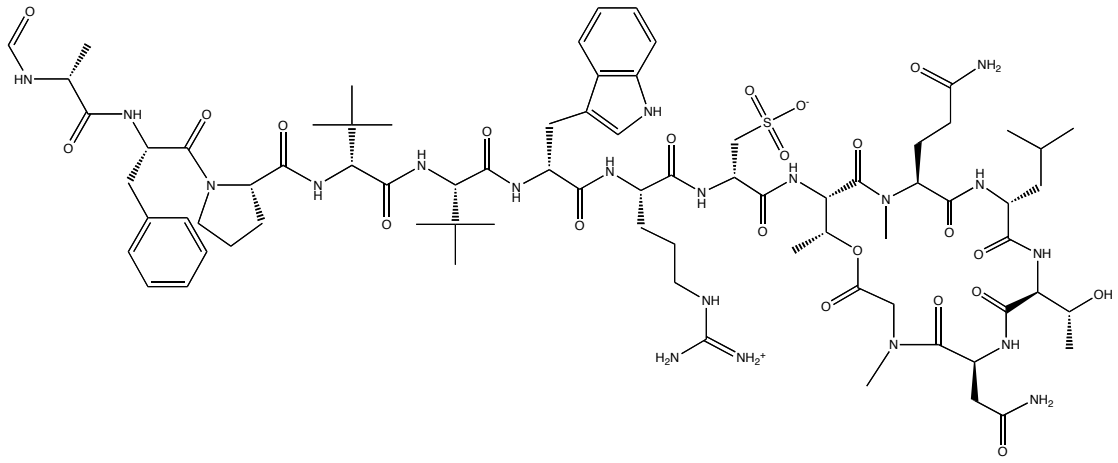


Figure 1-7 Structure of discodermin A originally isolated from *Discodermia kiiensis*.

The cyclic desipeptides jaspamides and geodiamolides (Figure 1-8) have been isolated from numerous tropical marine sponges and jaspamide (Zabriskie *et al.* 2002) was the first desipeptide reported. Geodiamolides A and B were originally isolated from *Geodia sp.* (Chan *et al.* 1987) and like many other desipeptides show encouraging cytotoxic activity. These compounds have been isolated from a large variety of parent sponge species leading to suggestions they may originate from microbial sources. This suggestion has been supported by the isolation of analogues of the jaspamides, the chondramides (Figure 1-8), from various cultures of Chondromyces (Kunze *et al.* 1995).

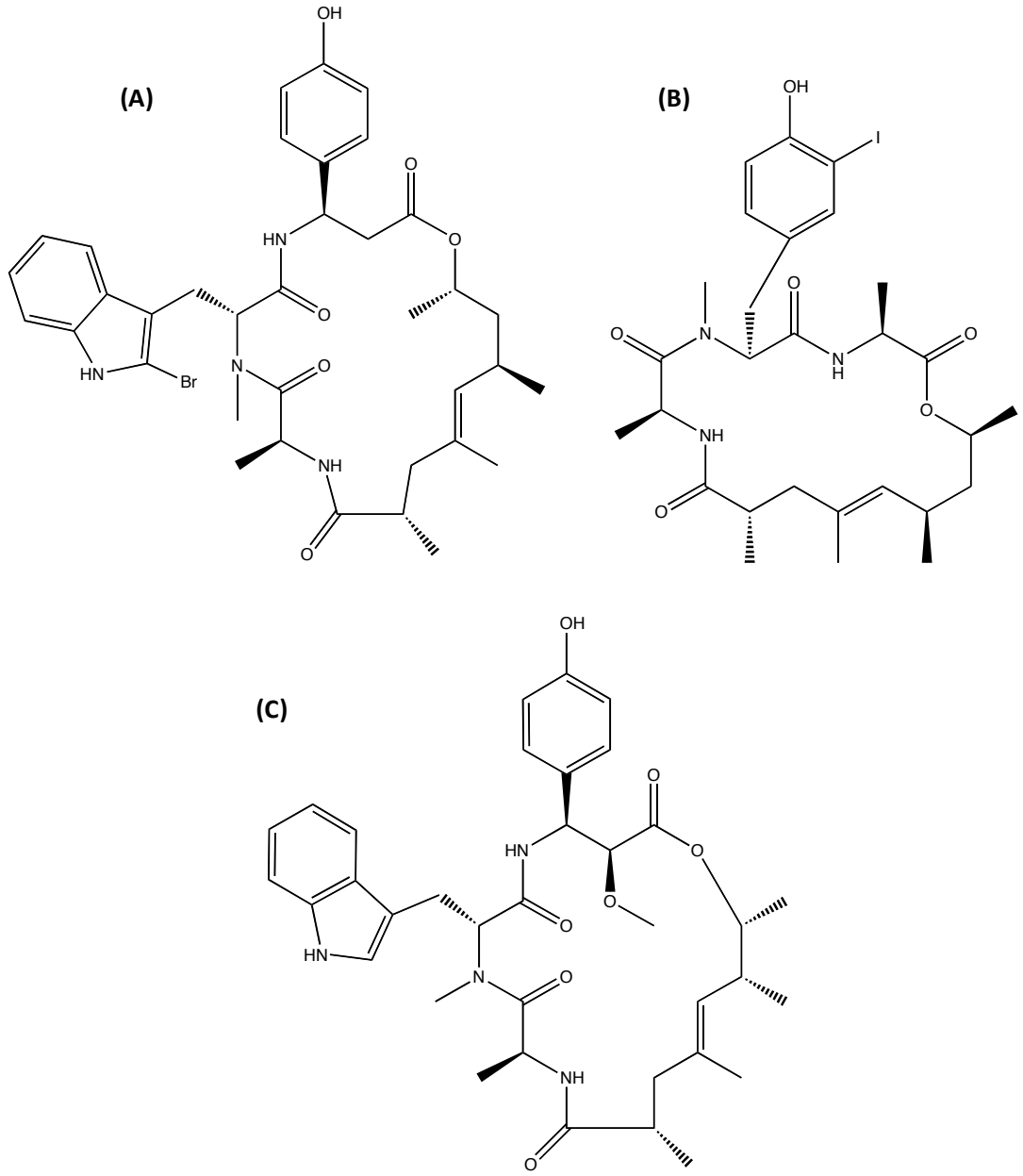


Figure 1-8 Structure of (A) Jaspamide, (B) Geodiamolide A and (C) Chondramide A.

More recently Dysinosins (Figure 1-9) originally isolated from the family Dysideidae (Carroll *et al.* 2002) have also shown cytotoxic activity and are structurally related to the aeruginosins originally isolated from a cyanobacterium. The hymenamides (Figure 1-9) (Kobayashi *et al*. 1993; Tsuda *et al.* 1993), originally isolated from the genera Hymeniacidon, and the structurally similar phakellistatins (Figure 1-9) are proline-rich cyclopeptides that also show cytotoxic activity.

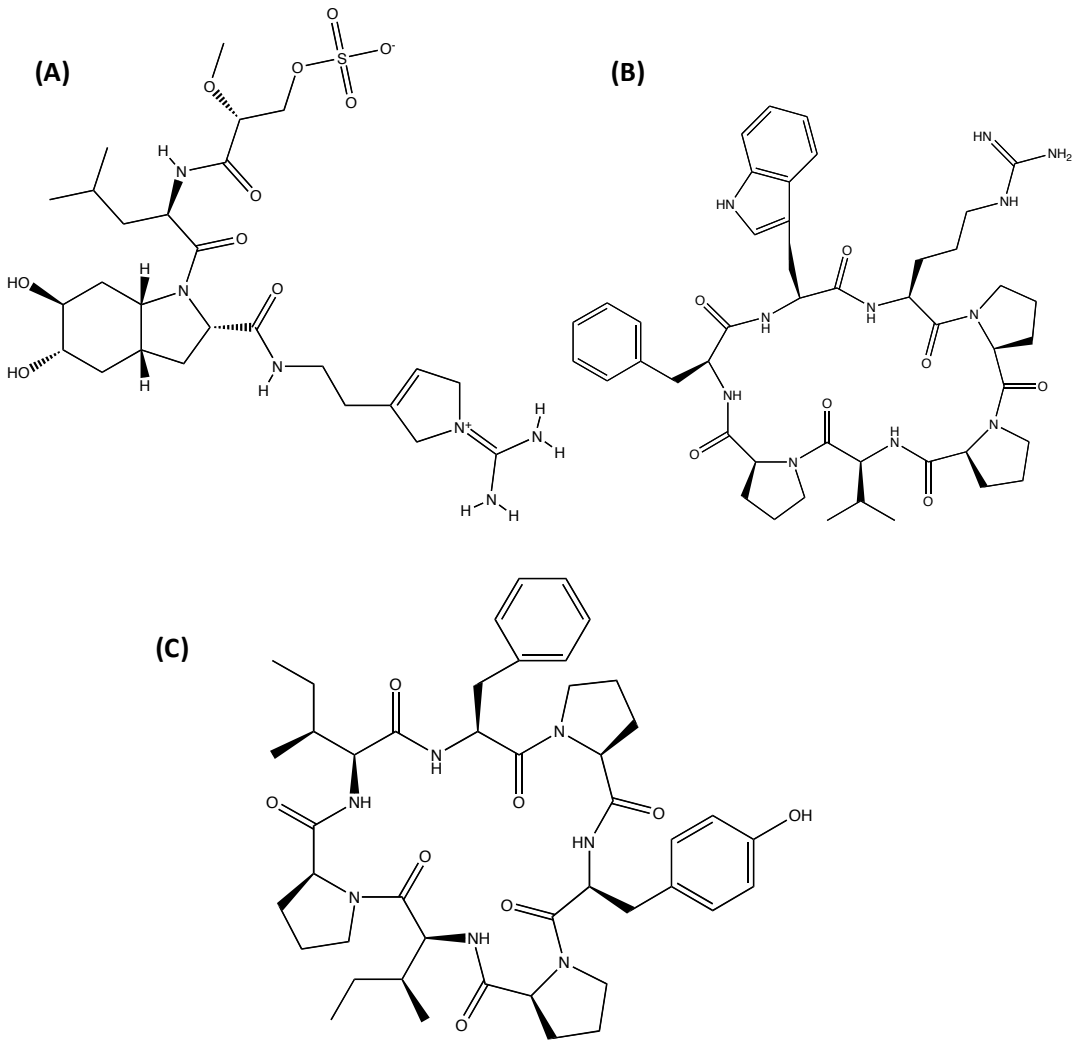


Figure 1-9 (A) Dysinosin A; (B), Hymenamide A; (C), Phakellistatin 1.

Many other bioactive peptides have also been isolated from marine sponges including the dolastatins, theonellamides, the axinstatins, the axinellins, the stylissamides, the mirabamides, the celebesides and the keramides, all of which show diverse complex structures (Fattorusso *et al. 2012)*.

1.4.2 Polyketides

Polyketides are secondary metabolites produced by a variety of living organisms including sponges. They represent another large class of bioactive compounds including the macrolide antibiotics which act by binding to the 50S subunit of rRNA during protein synthesis (Neal 2012).

Halichondrin B (Figure 1-10) is a bioactive macrolide originally isolated from the genus *Halichondria* (Hirata and Uemura 1986). The structure of eribulin mesylate (Y. Wang *et al.* 2007), which is now a licensed product (Figure 1-23), was developed from the macrocyclic ring found on halichondrin B.

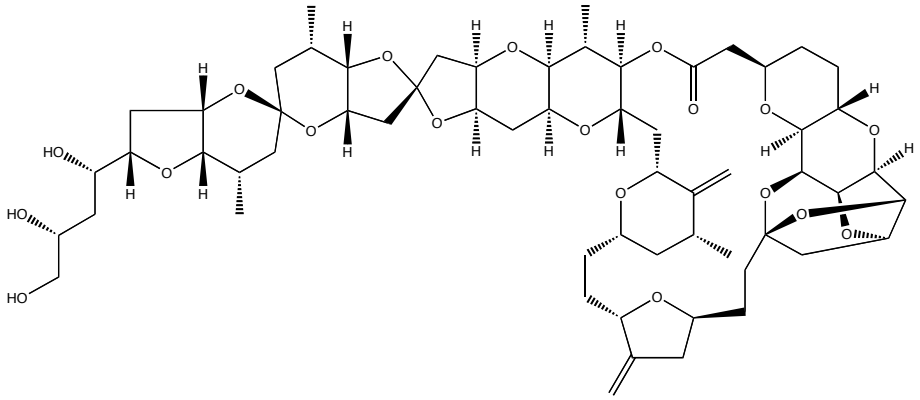


Figure 1-10 Structure of halichondrin B.

(+)-Discodermolide (Figure 1-7) is a potent antiproliferative and antimitotic compound originally isolated from the deep-sea marine sponge *Discodermia dissoluta* (Gunasekera *et al.* 1990). It has a distinctive structure through its linear backbone and displays a unique mechanism of action with negligible toxicity (Singh *et al.* 2008). Due to the relative poor yield of recovery from its source sponge (0.002%) and it only being found in sponges collected from greater than 35m (due to its light sensitivity), a significant effort was attempted to synthesise or semi-synthesize the compound. All attempts of semi-synthesis have proved unsuccessful (Singh *et al.* 2008) but the compound remains in clinical trials today, with production through a complex total synthesis process, with a yield of just 0.65%.

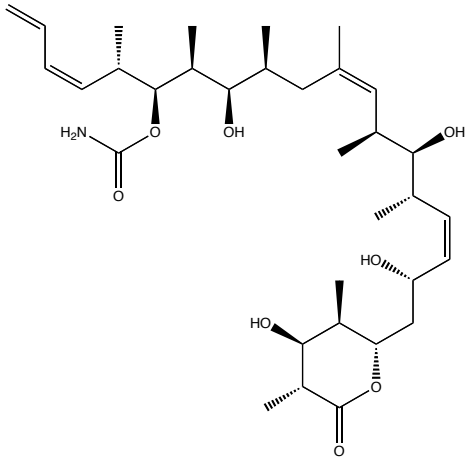


Figure 1-11 (+)Discodermolide displaying U shaped backbone.

Polyketides represent one of the most interesting classes of novel marine sponge derived secondary metabolites from a lead discovery perspective. However multiple polyketides isolated from a sponge are now known to be produced by associated microorganisms inducing swinholide A (Bewley *et al*. 1996), onnamide (Piel *et al*. 2004) and pysmberin (Fisch et al. 2009). Microorganisms were identified as the source of these compounds by genome mapping of both parent sponge and associated/symbiotic bacteria species. Another study has however shown that some parents sponge species may in some cases still be responsible for polyketide synthesis by running parallel sequencing of the polyketide biosynthetic genes of both parent sponge and microbial symbiotes (Sala *et al*. 2014).

1.4.3 Alkaloids

Alkaloids are one of the most studied and exploited classes of natural products and have been utilised throughout history including in 'modern medicine' (Figure 1-3). Marine sponges provide an interesting source of unique alkaloid structures and like other MNPs they often incorporate halogens due to their increased concentration in our oceans (Fattorusso *et al*. 2012).

Bromopyrrole alkaloids are found exclusively in marine sponges and oroidin (Figure 1-12), first isolated form *Agelas oroides*, is considered the major precursor of this group (Forenza *et al*. 1971). The pyrrole-imidazole functional group forms the identifying feature of the class and over 150 different compounds related compounds have since been identified. This class of alkaloid has been predominantly isolated form the families Agelasidae, Axinellidae and Halichondridae (Braekman *et al*. 1992) and have been found to produce a feeding deterrent effect (Lindel *et al*. 2000). Bromopyrrole alkaloids have attracted much interest from natural product researchers and synthetic chemists due to their significant pharmacological activity against a multitude of conditions including antimicrobial and cytotoxic activity.

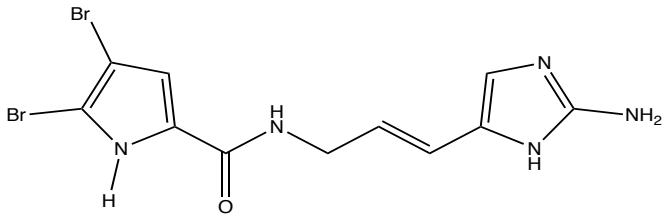


Figure 1-12 Structure of oroidin.

Initial differences in bromopyrrole alkaloids were identified with simple functional group changes and repositioning of the double bond in the amine side chain (Kobayashi *et al*.

1986). Small changes such as a hydroxyl switch on the amine side chain of dispacamide A showed a significant loss of activity (Cafieri *et al*. 1996). The oroidin skeleton can also be cyclized to form compounds such as hymenialdisine and debromohymenialdisine (Figure 1-13) both of which show potent antitumor activity as well as treatment potential against diabetes, cancer and Alzheimer's disease due to strong activity against several protein kinases (Martinez *et al*. 2002). Further cyclization of oroidin is seen in the structures of the phakellistatins, palau'amines and agelastatin A (Figure 1-13), which are bioactive against cancer cell lines (Pettit *et al.* 1997; Kinnel *et al.* 1998; D'Ambrosio *et al.* 1996)

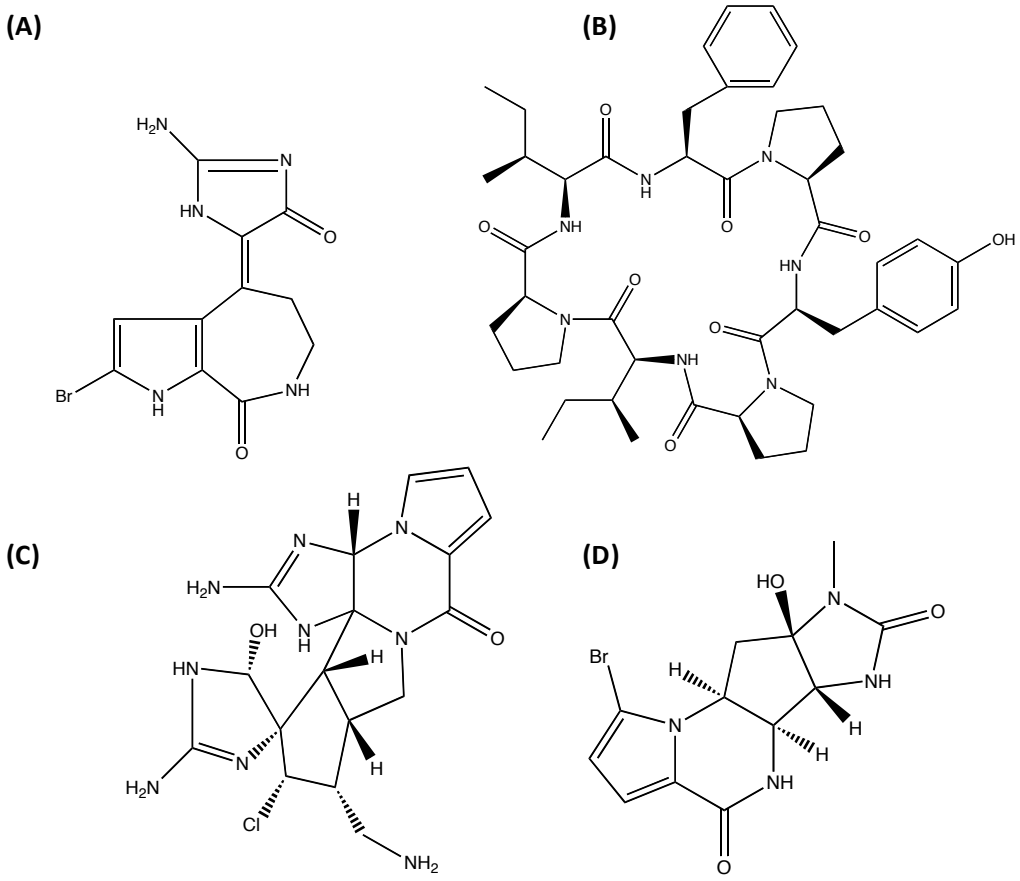


Figure 1-13 Structure of (A), Hymenialdisine; (B), Phakellistatin 1; (C), Palau'amine and (D) Agelastatin A.

Dimeric bromopyrrole alkaloids have also been isolated, the first of which was sceptrin (Figure 1-14), which was isolated form *Agelas sceptrum*. Sceptrin has been shown to display an expansive spectrum of activity including antimicrobial, antimuscarinic and antiviral (Bernan *et al*. 1993). Further dimeric bromopyrrole have also been isolated including the bioactive ageliferin and nagelamides (Fattorusso *et al*. 2012).

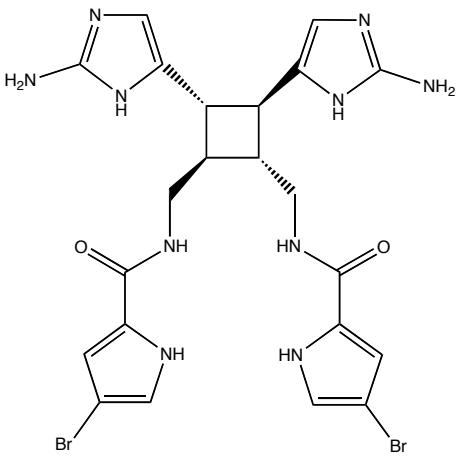


Figure 1-14 Structure of sceptrin.

Bromotyrosine alkaloids were first isolated from *Verongia aerophoba* in 1970 in the form of the antiproliferative (+) aeroplysinin-1 (Figure 1-15)(Fattorusso *et al*. 1970). This has since been isolated from multiple sponges of the order Verongida and genera *Jaspis* and *Poecillastra*. It is thought to be formed through enzymatic biotransformation of complex brominated isoxazoline alkaloids as a reaction to tissue damage (Thoms *et al*. 2006). In a similar manner to bromopyrrole alkaloids, bromotyrosine alkaloids show variation in the simple structure of (+) aeroplysinin-1 with analogues synthesized to increase activity. These alkaloids also show a similar increase in complexity in natural analogues such as the symmetrical psammaplin A (Figure 1-15), which has also been isolated from sponges of the order Verongida and has shown activity against multiple human cancer cell lines and antimicrobial activity (Rodriguez *et al*. 1987; D. Kim *et al*. 1999). Dimeric and cyclic analogues of psammaplin A have also been isolated from sponges of the same order and genera as more simple bromotyrosine alkaloids (Park *et al*. 2003).

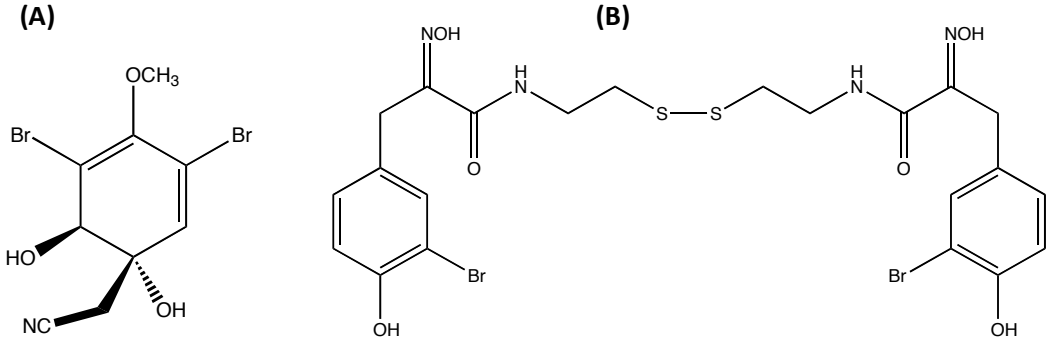


Figure 1-15 Structures of (A), (+) aeroplysinin-A and (B), Psammaplin A.

Purealidins, purpurealidins and bastadins represent three more classes of bioactive bromotyrosine alkaloids. Eighteen different purealidins have been identified and are most

commonly isolated from *Psammaplysilla purea* although other purealidins have been isolated from *Druinella sp.*(Tabudravu and Jaspars 2002). Purealidin A (Figure 1-16) was the first bromotyrosine alkaloid of this class identified and represents the model compound of the class which are known to show cytotoxic and antibacterial activity. Purpurealidins (Figure 1-16), also isolated form *Psammaplysilla purea*, have similarly displayed strong antibacterial activity and their suggested ecological role is as feeding deterrents (Koulman *et al*. 1996). Bastadins (Figure 1-16) are similarly commonly isolated from the genus Verongida and are formed as heterodimers from the combination of two brominated tyrosine–tyramine amides. They can be structurally classified into three different groups (Jaspars *et al*. 1994) and 24 analogues have been isolated from marine sponges. The first bastadins were isolated from *Ianthella basta* and have been shown to display potent antimicrobial and cytotoxic activity (Carney *et al*. 1993; Kazlauskas *et al*. 1981).

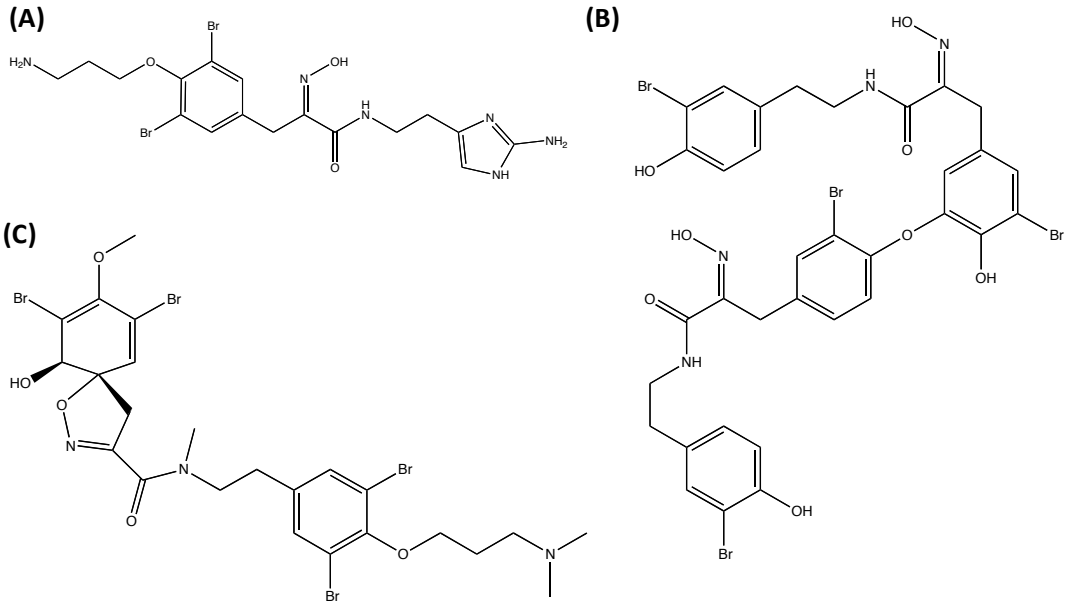


Figure 1-16 Structure of (A) Purealidin A; (B), Purpurealidin A; (C), Bastadin 1.

The manzamine alkaloids are a further class of marine sponge alkaloids and are identified by their polycyclic beta-carboline structure. Manzamine A (Sakai *et al.* 1986), which was first isolated from the genus *Haliclona*, has displayed cytotoxicity activity against leukaemia. Since the isolation and identification of manzamine over 50 analogues have been identified from multiple genera of sponge, which has led to suggestions of potential microbial origin (Konig *et al*. 2006). Since isolation and identification of further manzamine alkaloids they have been shown to be an extremely bioactive class and have displayed cytotoxic,

antimalarial and antibacterial activities (Sakai *et al.* 1986), they have also now been isolated directly from bacteria (J.-F. Hu *et al*. 2003).

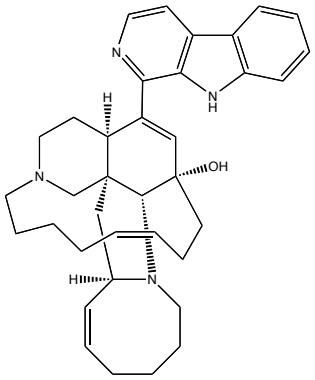


Figure 1-17 Structure of manzamine A.

1.4.4 Terpenes

Terpenes are considered to be both primary and secondary metabolites of marine sponges and are assembled from five isoprene units resulting in a large variety of structures and bioactivity.

Sesterterpenes include the compound manoalide (Figure 1-18), which was first isolated from *Luffariella variabilis*. Manoalide is known to display a range of bioactivity, including antibacterial activity, and reached phase II clinical trials as an anti-psoriatic. It exerts its action as an irreversible binder of PLA2, while an analogue, luffareiolide (Figure 1-18), binds reversibly. Luffereiolides, also first isolated from *Luffariella variabilis*, have been shown to exhibit potent cytotoxic activity and further analogues, isolated from the same sponge, the luffarelins have been shown to have antibacterial activity. More complex bicyclic sesterterpenes have also been isolated from sponge sources, including palauolide (Figure 1-18), the thorectandrols and the petrosaspongiolides all of which display activity against cancer cell lines.

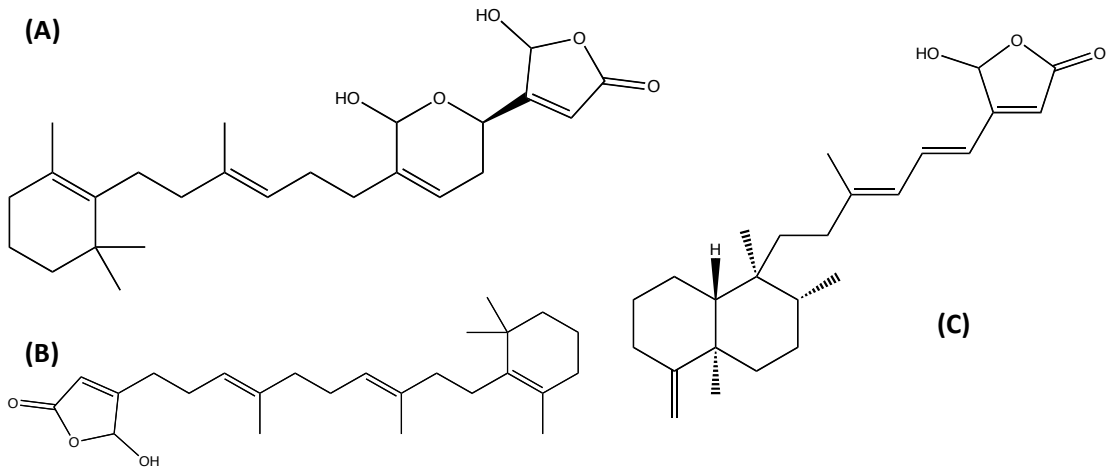


Figure 1-18 Structure of (A), Manoalide; (B), Luffereiolide and (C), Palauolide.

Triterpenes were the first marine isoprenes discovered and research has continued into the class due to their broad spectrum of bioactivity and diverse structures. Two significant classes of triterpenes are the isomalabaricane triterpenes and the steroidal saponins (Fattorusso *et al.* 2012). The malabaricanes are not unique to marine sponges and have been isolated from terrestrial sources but isomalabaricanes (characterised by its trans-syn-trans ring junction) were first isolated from *Jaspis stellifera*. The isomalabaricanes include the stelletins, the rhabdastrellins and the stelliferins, with some compounds within each class displaying antiproliferative activity (Ravi *et al*. 1981; Kobayashi *et al*. 1996; Lv *et al*. 2008). Further differentiation within the isomalabaricanes separates other isolated compounds into nortriterpenoids, norseterterpenoids and norditerpenoids, these include the jaspiferals, the aurorals and the jaspolides which also display potent cytotoxicity (Kobayashi *et al*. 1996; Tang *et al*. 2006; Bourguet-Kondracki *et al*. 2000).

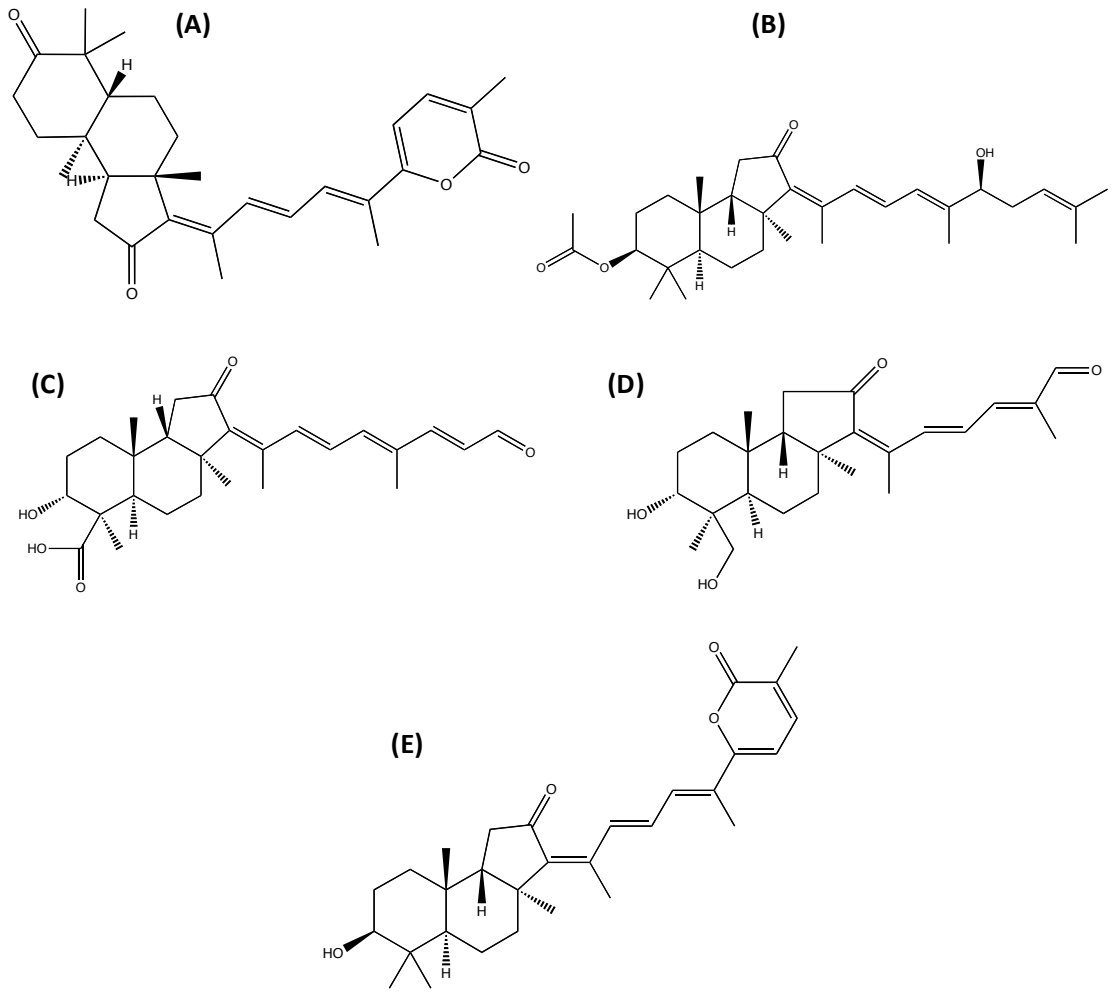


Figure 1-19 Structure of (A), Stelletin A;(B), Stelliferin B;(C), Jaspiferal A; (D), Auroral 1 and (E), Jaspolide A.

Steroidal saponins are commonly associated with other marine invertebrate such as starfishes but have also been isolated from marine sponges. Eryloside A (Figure 1-20), first isolated from the family Geodiidae, has been shown to have antitumor and antifungal activity (Afiyatullov *et al*. 2007). Formoside A (Figure 1-20) was first isolated from *Erylus formosus* and is known to deter predatory fish (Jaspars and Crews 1994; Kubanek *et al*. 2000).

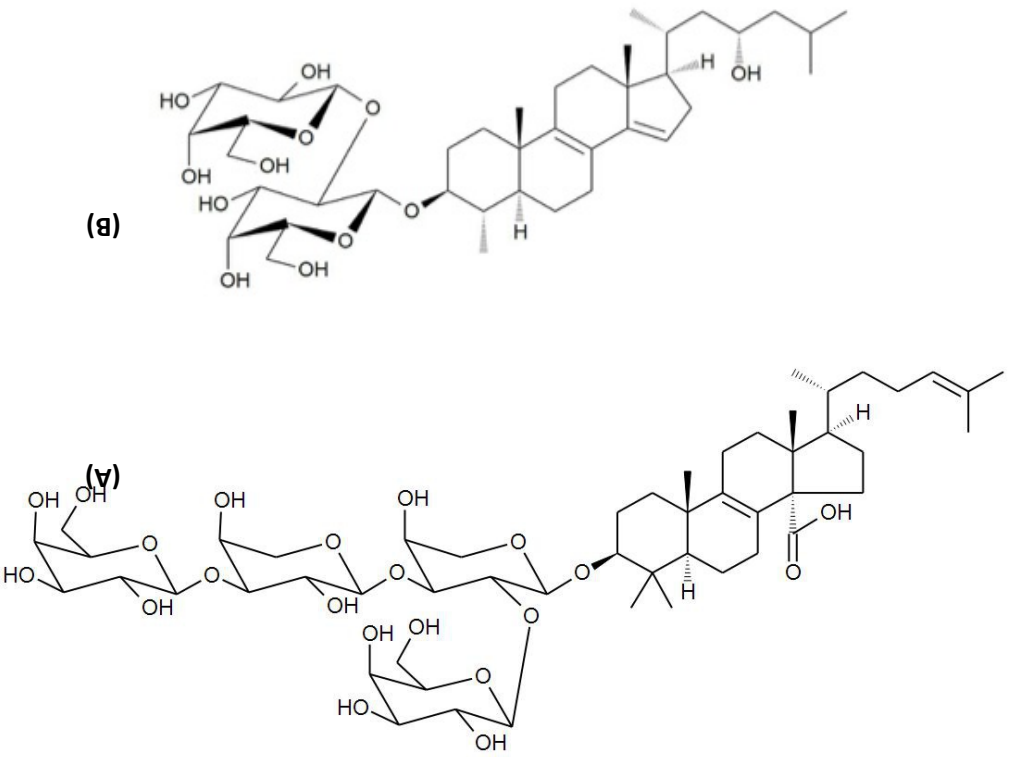


Figure 1-20 Structure of (A), Eryloside and (B) Formoside.

Many different reasons have been suggested for the incredible variety of bioactive MNPs isolated from sponges. These are mainly ecological roles such as defence against predators including sea slugs and nudibranchs (Proksch 1994) and spatial competition with other sponges or species, fouling organisms and microbes (Taylor *et al*. 2007; Jackson and Buss 1975). Numerous studies have aimed to determine relationships between sponge secondary metabolite production and factors such as their location (Green 1977), seasonal trends (Sacristán-Soriano *et al*. 2012) and spatial scales (Sacristán-Soriano *et al*. 2011). A study completed in the Americas looking at sponge species found between Washington, USA and Vera Cruz, Mexico showed an increase in toxin production from north to south. This was determined by pipetting sponge extracts into tanks containing goldfish and observing if the fish subsequently perished (Green 1977). The reasoning for this increased production of toxic metabolites is believed to be correlated to the rise in diversity and density of fish species causing more competition for food, making it more likely for fish to graze on sponge and other marine invertebrates (Green 1977). What these individual studies highlight is that numerous factors play a role in the chemical diversity found in sponges and the details

accounting for such individual variations are not fully understood and are difficult to quantify in uncontrolled environments.

1.4.5 Licensed medicines derived from marine sponges.

As the majority of antibacterial and anticancer drugs are derived from nature (Figure 1-4), researchers are always searching for unique conditions to find innovative and exciting leads and unstudied sponges in alternative environments could potentially provide this. Technological advances in deep-sea collection have aided sampling and supply problems, which have historically hindered collection, so the number of sponge derived natural products may increase substantially over the coming years (Laport *et al*. 2009). Thousands of different species of sponge have been identified and they are the most common source of marine derived natural products (Figure 1-6), but currently only three marine sponge derived products have FDA and MHRA approval (Figure 1-21**,** Figure 1-22**,** Figure 1-23) (Hill and Fenical 2010; Kingston 2009).

1. Cytarabine (Ara-C) (Figure 1-21)- Derivative from sponge natural product. A nucleoside, targets DNA polymerase in cancer cells.

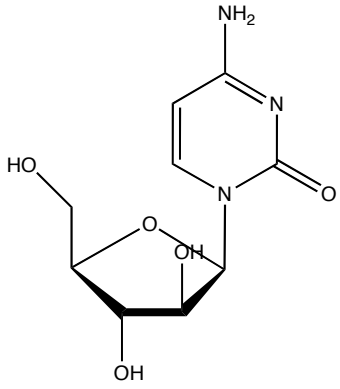


Figure 1-21 Structure of cytarabine; Formula: $C_9H_{13}N_3O_5$; Mass = 243.09

2. Vidarabine (Ara-A) (Figure 1-22)- Derivative from sponge natural product. Antiviral nucleoside targeting viral DNA polymerase 1.

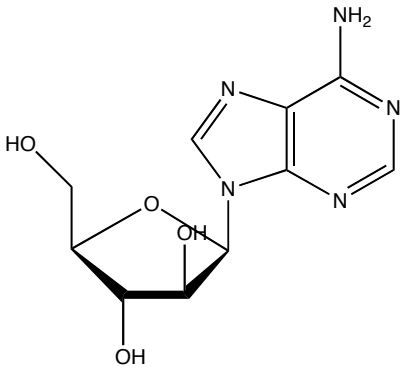


Figure 1-22 Structure of vidarabine; Formula $C_{10}H_{13}N_5O_4$; Mass 267.10.

3. Eribulin mesylate (Figure 1-23)- Derivative from sponge natural product (Zheng *et al*. 2004). Complex polyketide, which targets microtubules in cancer.

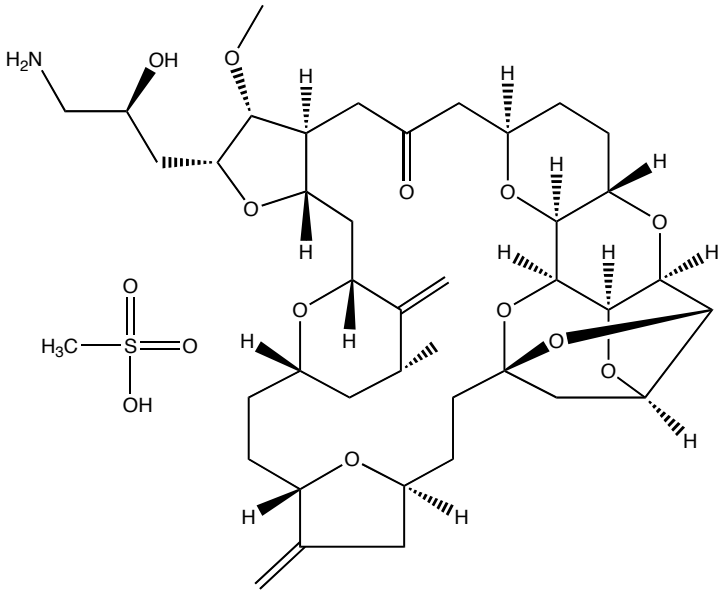


Figure 1-23 Structure of eribulin mesylate; Formula: $C_{41}H_{63}NO_{14}S$; Mass 825.40

1.4.6 Secondary metabolite biosynthesis: is it the bacteria or the sponge?

Bacteria can contribute up to 50 % of a sponge's biomass (Grozdanov and Hentschel 2007), therefore it would be fair to hypothesise they play a large part in the production of any metabolites extracted from a sponge mass (Lee *et al*. 2001). There is still however a lack of understanding about the relationships formed between sponges and the bacteria found on them, although much current research is looking at sponge associated bacteria with publications increasing significantly over the past 25 years (Figure 1-24 and Figure 1-25).

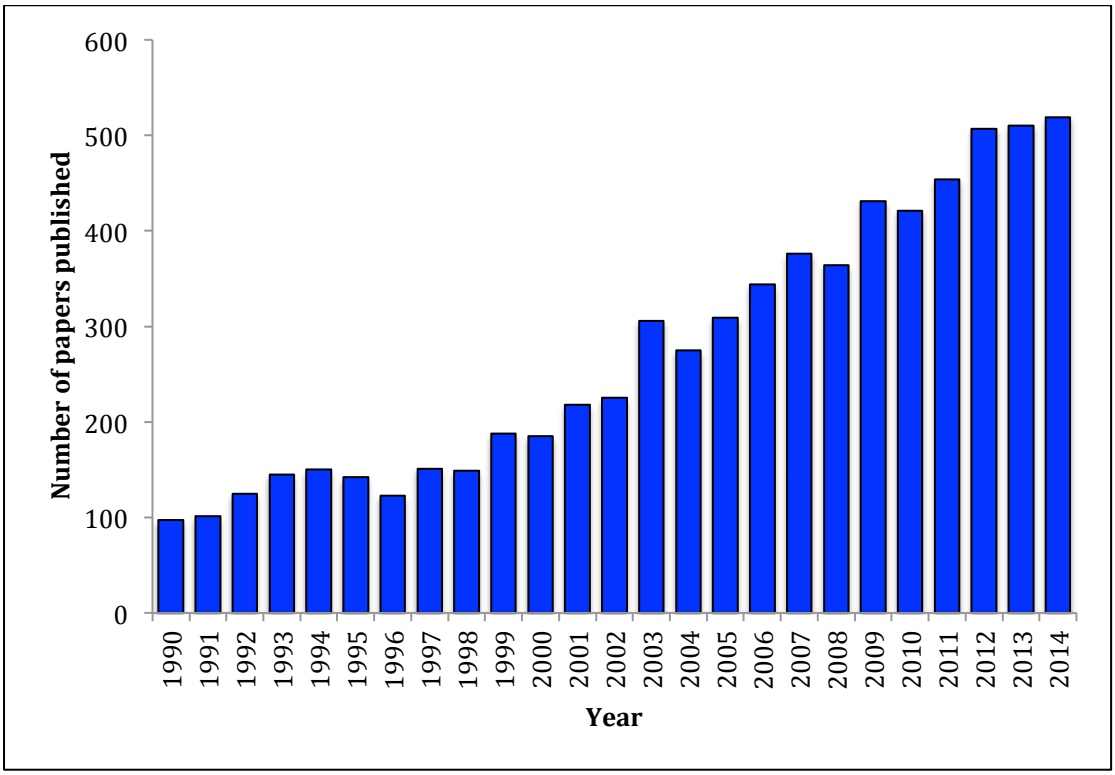


Figure 1-24 Increasing research interest in marine sponge-microorganism associations. Number of publications retrieved from the ISI Web of Science database by using the following search string: (sponge* or porifera* or demospong* or sclerospong* or hexactinellid*) and (bacteri* or prokaryot* or microbe* or microbial or microorganism* or cyanobacteri* or archaeon or archaea* or crenarchaeo* or fung* or diatom* or dinoflagellate* or zooxanthella*) not (surgery or surgical). Not surgery or surgical was added to stop the identification of sponges in surgery.

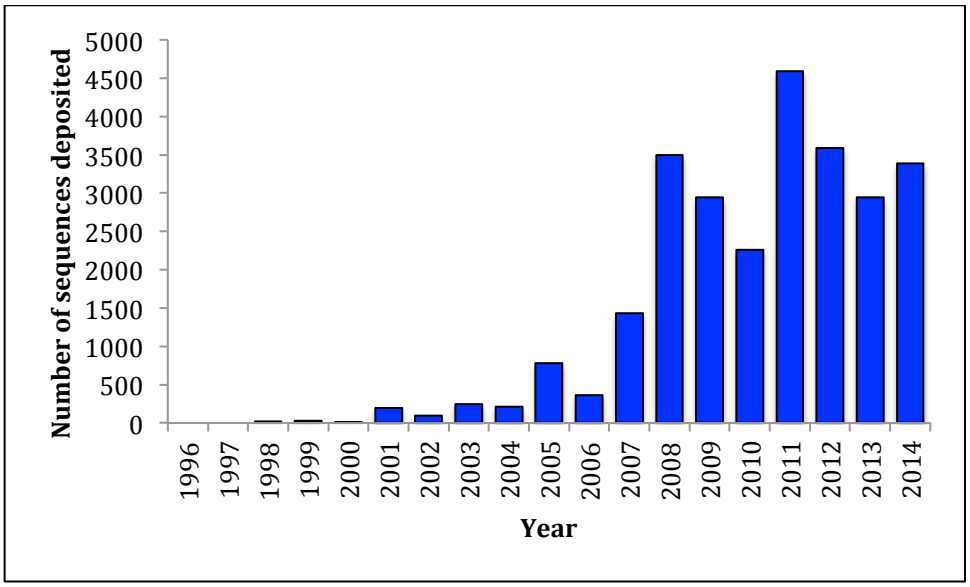


Figure 1-25 Number of sponge-derived 16S rRNA gene sequences deposited in GenBank per year. The following search string was used. (sponge* or porifera*) and (16S* or ssu* or rRNA*) not (18S* or lsu* or large subunit or mitochondri* or 23S* or 5S* or 5.8S* or 28S* or crab* or alga* or mussel* or bivalv* or crustacea*).

The hypothesis that bacteria play a significant role in the production of secondary metabolites is reinforced by multiple studies in which sponge-associated bacteria have been cultivated and screened for the production of active metabolites (Grozdanov and Hentschel 2007; Flemer *et al*. 2012; Abdelmohsen *et al*. 2010). This is also noted when looking at the structures of compounds isolated from sponges (section 1.4) notably the non-ribosomal peptides and polyketides (Faulkner *et al*. 1994; Waters *et al*. 2010; M. C. Wilson *et al*. 2014). An example of one the first cases of clear evidence of associated bacteria being the true origin of a sponge isolated secondary metabolite came in the form of the chlorinated metabolites previously thought to be produced by *Dysidea herbacea*, but actually produced by a marine cyanobacterium (Unson and Faulkner 1993). This could theoretically dismiss one of the significant potential limitations of marine sponges in that the secondary active metabolites are usually produced in relatively small amounts with the sponge as a primary resource (Belarbi *et al*. 2003; Skropeta 2008).

As the yield of MNP's from sponge material is so low and collection from natural habitats is not sustainable the previous alternatives were to either cultivate sponges (Klöppel *et al*. 2008) and extract the natural product, or synthesise the natural product (Richards *et al*. 2008). Cultivation of some marine sponge species is possible in aquaculture and on sponge farms and represent a primary method of producing natural bath sponges (Duckworth 2009). Sponge farming has more recently been utilised as a source of the bioactive metabolite

halichondrin B from its host sponge (Munro *et al*. 1999). *Aplysina aerophoba* has also been successful cultivated in aquaculture with attempts to mimic in situ environments (Klöppel *et al*. 2008). However, farming and aquaculture of sponges is often complex and could potentially alter the bacterial community present on a sponge, as the bacteria may only be present due to the season, spatial competition from other species or the surrounding environment (Klöppel *et al*. 2008). The other option would be undertake total synthesis of the compound or design and synthesise an analogue (Zheng *et al*. 2004; Richards *et al*. 2008). In reality this is not always possible, but attempts to create synthetic analogues will always address pharmaceutic considerations to create the most effective and safest drug possible.

If the natural product required is found to be of bacterial origin, it may be possible to cultivate the bacterial species or use recombinant technology to transfer the biosynthetic genes into a culturable host to produce metabolites in laboratory conditions (Hentschel *et al*. 2012). This is a common method already exploited by pharmaceutical companies for modern medicines, such as insulin and can make the yield from any extraction much greater. Any initial harvesting of sponge is kept to a minimum allowing the scientist to sustainably source new sponges whilst keeping good relations with conservationists who may help them with collection and identification.

It is thought the relationship between a sponge and their associated bacteria, may be symbiotic, as it appears to be a mutually beneficial scenario (Lee *et al*. 2001; Althoff *et al*. 1998; Balskus 2014; Freeman and Thacker 2011). The sponge gains food from the bacteria and a possible chemical protection and in return it provides the bacteria with protection, a chance to multiply and a source of free flowing food (Lee *et al*. 2001). It is worth considering however that not all species of bacteria found on a sponge will be symbiotic and some may even be pathogenic and damaging to the sponge (Taylor *et al*. 2007). Associations between sponge and bacteria can occur in two different ways, it is either through luck: *i.e.* the microbes are recruited from the surrounding environment; or it is possible they are symbiotes passed on through reproduction and growth (Taylor *et al*. 2007).

1.5 'Super bugs' or drug resistant bacteria

In 2013 Sally Davies the Chief Medical Officer of the UK issued a statement describing antibiotic resistance as a 'ticking time bomb' and 'as big a risk as terrorism.' She also

emphasised that if we don't take action we will "regress back to a time before antibiotics, where people could not have a simple operation without risk of dying" (Davies *et al*. 2013). This is a problem increasingly in public eye with the UK Prime Minister David Cameron publicly highlighting it and the award of a national research award, the Longitude Prize (Burki 2014; Rees 2014). Until 2015, no new class of antibiotics had been successfully developed since the early 1980s with few antibiotics progressing into the robust stages of development. However, in 2015 teixobactin a new, structurally novel, peptide antibiotic was discovered, which inhibits cell wall synthesis by binding to a precursor of peptidoglycan. Highly significantly, it shows no detectable development of resistance, potentially representing a novel mode of action (Ling *et al*. 2015). The innovative approach to the discovery of teixobactin, from a natural resource, validates the resurgence of natural product research over synthetic approaches to drug discovery (Ling *et al*. 2015). The reasoning behind the previous failures to introduce new anti-microbials to the market is mainly due to the lack of profit associated with antibiotics and the huge costs associated with developing new medicines.

1.5.1 Classification of bacteria

Bacteria can be split into two distinct classes based on their cell envelope (Figure 1-26**)**. These classes are Gram-positive bacteria and Gram-negative bacteria. Gram-positive bacteria include the clinically relevant bacterium of methicillin resistant *Staphylococcus aureus* (MRSA) (Dunlap 2007) and *Clostridium difficile* (O'Neill 2014). Gram-negative bacteria include the bacterium *Escherichia coli*, which is currently becoming more clinically relevant due the steady rise in infection and resistance (O'Neill 2014). These two classifications of bacteria can easily be differentiated in the laboratory using Gram staining (Halebian *et al*. 1981) and the standard treatment for infection is usually different due to the structural differences of their cell envelopes (Halebian *et al*. 1981) (Figure 1-26).

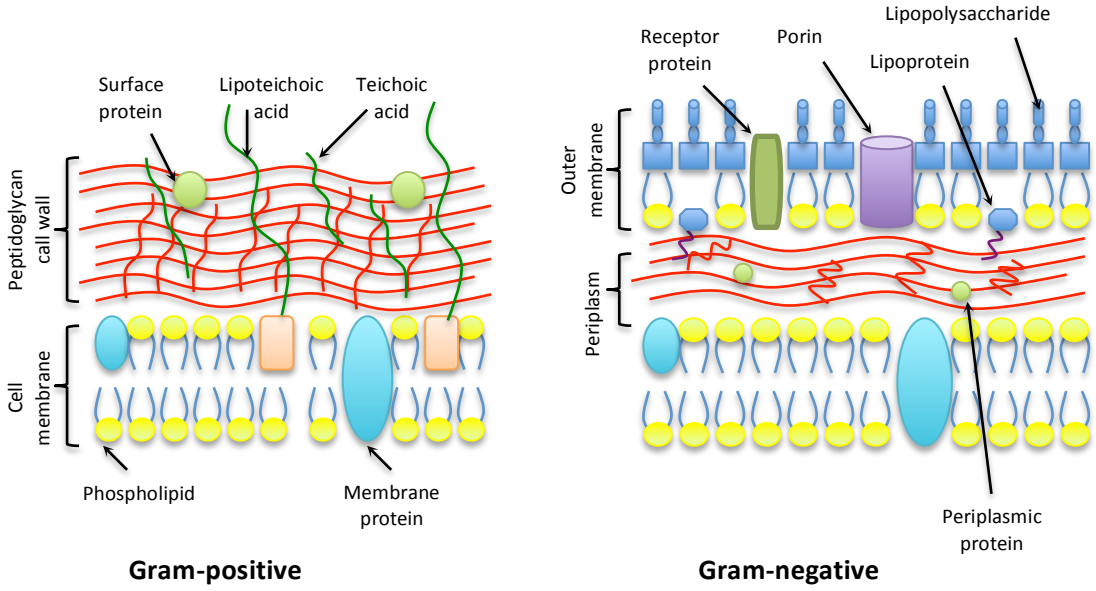


Figure 1-26 Cross sectional diagram of the cell envelope of Gram-positive and Gram-negative bacteria. Image adapted from Denyer *et al* (2011).

1.5.2 Anti-infective agents

An infection occurs when a foreign species invades the body and multiplies to form a colony at the expense of the host (Stedman's 2009). An anti-infective is something that acts against this harmful colonisation thus the term anti-infective encompasses all antiviral, antibacterial, antifungal and antiprotozoal drugs. Anti-infective agents can exhibit their action by killing the organism or stopping the spread of an organism; in the case of bacteria these different mechanisms of action are classified as bactericidal and bacteriostatic agents respectively.

1.5.3 Current classes of antibiotics

Antibiotics can be grouped into classes based on their mechanism of action and structure and these can be further narrowed by their general targets within a bacterial cell. Antibiotics target processes and structures within a microbial cell that are not present in mammalian cells effectively working as 'magic bullets.'

1.5.3.1 Protein synthesis inhibitors

The essential process of protein synthesis occurs in ribosomal subunits, which are of a different size in bacteria (30S and 50S) to mammalian cells (40S and 60S). Synthesis occurs within ribosomes by decoding messenger RNA (mRNA) and forming proteins using transfer

RNA (tRNA). Synthesis happens in three steps; delivery of tRNA to the ribosome, transpeptidation of amino acids across the P and A site of the ribosome following reading of mRNA and translocation of the protein to the P site (Neal 2012). Different classes of antibiotics target this process at different points (Neal 2012). Tetracyclines block tRNA binding, oxalizidonones (*e.g.* Linezolid) blocks the initiation of the mRNA/RNA complex, aminoglycosides (*e.g.* Gentamicin) cause incorrect reading of mRNA, chloramphenicol inhibits transpeptidation and macrolides (e.g. erythromycin) and streptogramins inhibit translocation (Neal 2012).

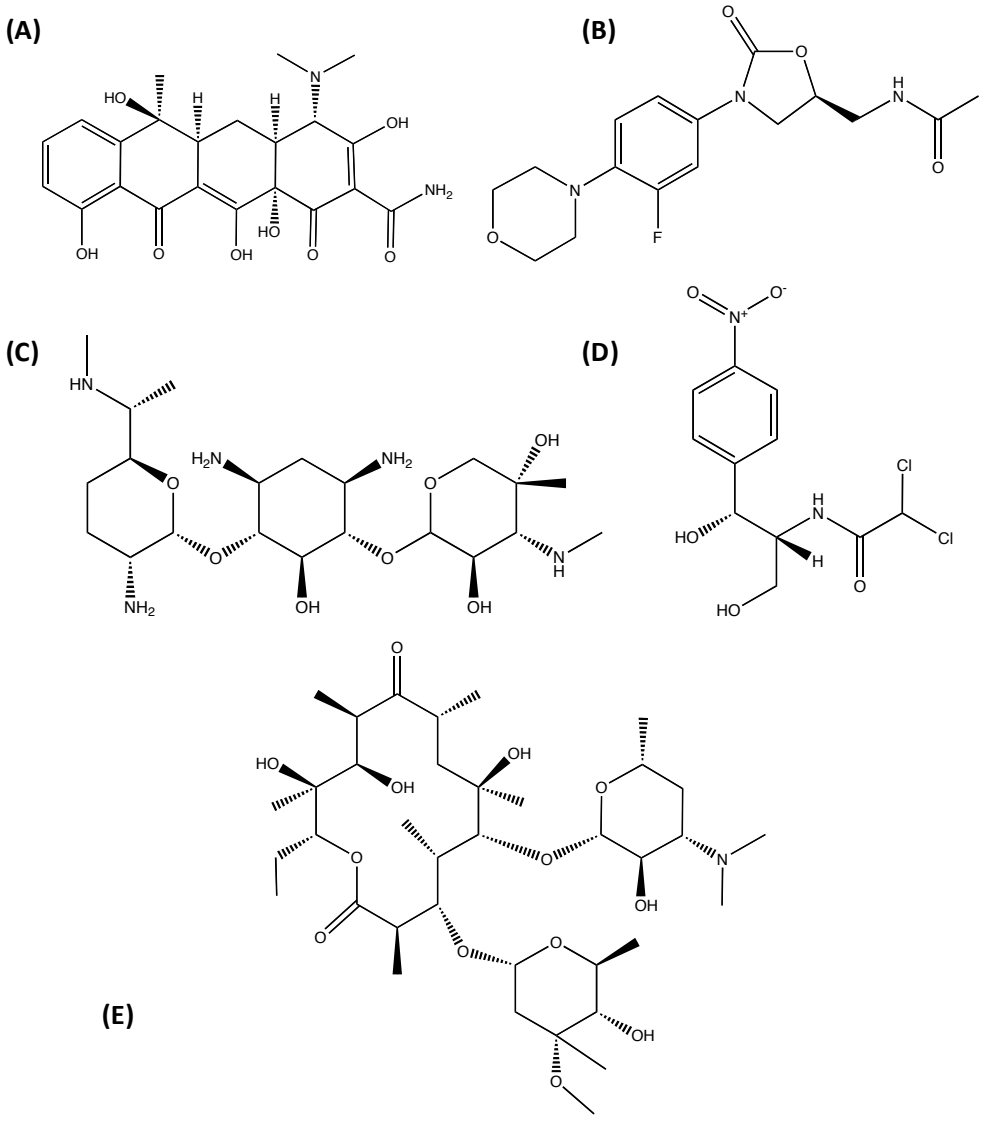


Figure 1-27 Structure of (A), Tetracycline; (B), Linezolid; (C), Gentamicin; (D), Chloramphenicol and (E); Erythromycin.

1.5.3.2 Cell wall synthesis inhibitors

The bacterial cell wall is different to that of mammalian cells (section 1.5.1) making unique structures found in bacterial cell walls, such as peptidoglycan, excellent targets for bacterial specific modes of action. Beta lactams (e.g. Amoxicillin) and the closely related cephalosporins (e.g. cefuroxime) are bactericidal and act by inhibiting peptidoglycan transpeptidation, which is critical for the stability of bacterial cells (Neal 2012). Essential to the activity of both of these classes is the stability of the beta-lactam ring, which mimics the structure of the peptide chains of the bacterial cell wall. Some bacteria have subsequently developed resistance to beta-lactams through the production of penicillinase, which denatures the beta lactam ring, this has been overcome by synthetic changes. Vancomycin also inhibits peptidoglycan formation by blocking the ends of the pentaglycine bridge (Neal 2012).

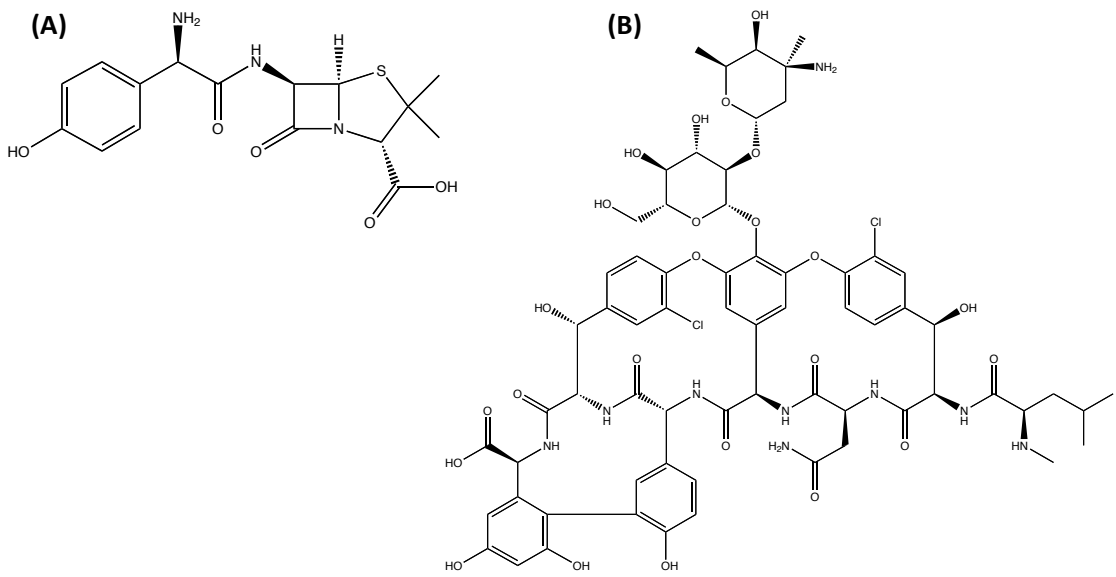


Figure 1-28 Structure of (A), Amoxicillin and (B) Vancomycin.

1.5.3.3 Nucleic acid synthesis inhibitors

Folic acid is essential for the production of purines and pyrimidines and therefore the production of DNA and unlike eukaryotes bacteria biosynthesize folic acid within the cell whereas eukaryotes need to ingest folic acid as part of their diet (Neal 2012). For this reason folic acid production is a good target for antibacterials and the sulphonamides (e.g. sulfadiazine (Figure 1-29)) were the first class of antibacterial found to be effective against systemic infections. Sulphonamides are analogues of p-aminobenzoic acid and inhibit dihydropteroate synthetase early in folic acid production. Trimethoprim (Figure 1-29) acts

against dihydrofolate reductase, which converts dihydrofolic acid to tetrahydrofolic acid (Neal 2012). The quinolones (e.g. Ciprofloxacin (Figure 1-29)) act by prevention of DNA supercoiling, the 5-nitromidazoles (e.g. metronidazole (Figure 1-29)) inhibit DNA synthesis following the reduction of the nitro group and rifampicin (Figure 1-29) prevents RNA transcription by inhibiting DNA polymerase (Neal 2012).

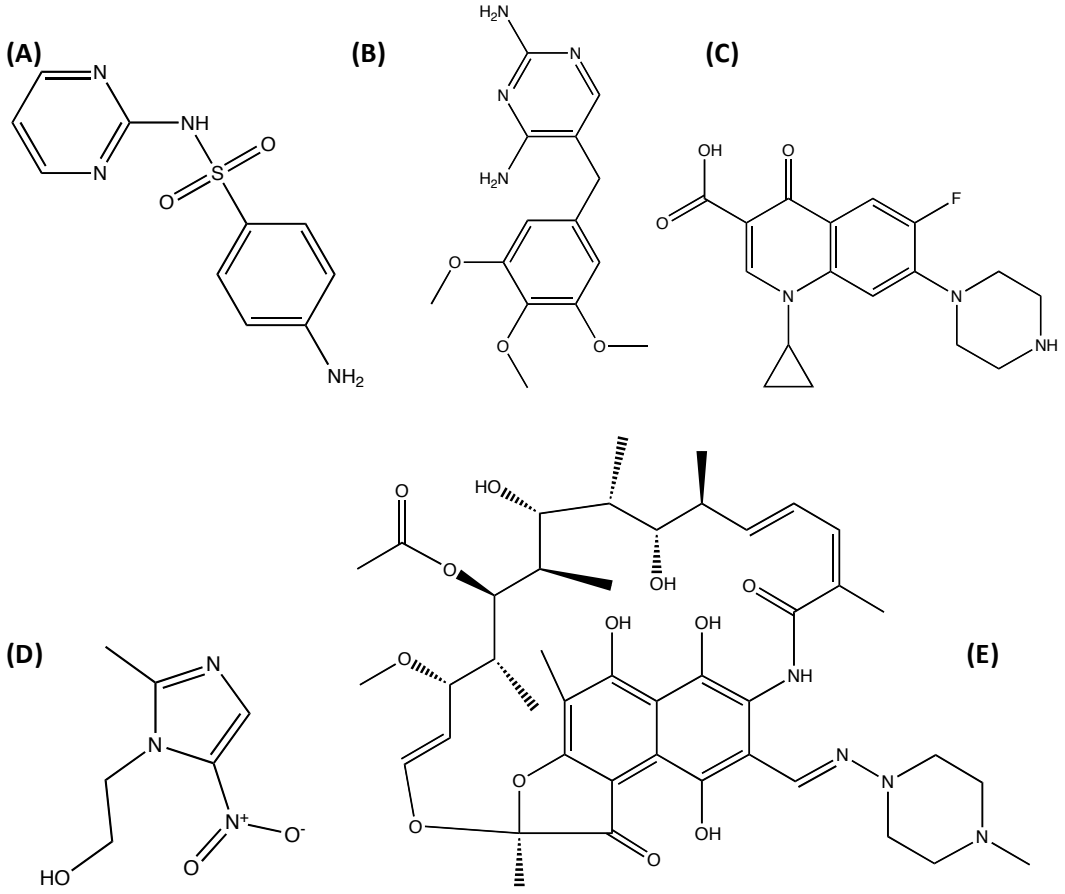


Figure 1-29 Structure of (A), Sulfadiazine; (B), Trimethoprim; (C), Ciprofloxacin; (D), Metronidazole and (E) Rifampicin.

1.5.4 The development of antibiotic resistance

The resistance of a microorganism describes its ability to remain unaffected by pathogenic agents (Stedman's 2009) these are often referred to in the media as 'super bugs' (Davies *et al.* 2013). The simplest way resistance arises is when a random genetic mutation occurs, during replication within a cell within a colony (Denyer *et al.* 2011). This often arises because of the rapid replication of bacteria and may spread to other local bacteria *via* plasmids. Random mutation may allow the cell to survive exposure to an anti-infective agent leaving just a resistant strain of a micro-organism now able to multiply (Aminov and Mackie 2007).

Resistance arises rapidly and spreads quickly, especially when plasmid transfer occurs (Hall and Barlow 2004). Resistance is less commonly transferred between fungal cells in comparison to bacteria, although it is possible (Norrby *et al.* 2005).

Resistance can also occur due to microorganisms forming a biofilm, which are notoriously difficult to treat and often require surgical removal (Denyer *et al*. 2011). For a biofilm to form an organic layer needs to be present on the infection site surface. This surface can be either biological tissue or a non-biological solid. A biofilm is thought to form in two steps; first cells attach themselves to a solid substrate and they then attach themselves to one another (Cramton *et al.* 1999). A single cell is much more susceptible to anti-infective treatment than a cell living in a biofilm, therefore cells within a biofilm are much more resistant to treatment (Denyer *et al*. 2011). There are various reasons for this development and they can be broadly split into a few major areas. Firstly, resistance can occur as cells, which are part of a biofilm, produce an extracellular polymeric substance (EPS) matrix; this may reduce the ability of an anti-infective to penetrate the biofilm (Denyer *et al.* 2011). Cells deep inside the biofilm do not absorb as many nutrients as those on the surface meaning their growth rate is reduced. Unfortunately the replication and growth of a cell is the primary target of many anti-infective agents, making these cells resistant to therapy by any bacteriostatic agents. Cells living in a biofilm, are so close to each other they should be able to exchange plasmids much more easily than singular cells (Denyer *et al.* 2011). This means the ability to combat immune responses and anti-infective agents might be capable of transfer from one cell to another through the exchange of genetic information. Cells may also be able to disguise themselves to the host and anti-infectives by mimicking local tissue using EPS (Denyer *et al.* 2011). As resistance is becoming more common, the number of treatments we have available to treat infections is reducing. Strains of bacteria have been found that are resistant to almost all anti-bacterials available (Bax *et al.* 2000; Johani *et al.* 2010; Davies *et al. 2013)* making management almost impossible.

1.5.5 Identifying the need for new antibiotics

The resistance problem escalated over the 1990s reaching its peak in the mid-2000s (Figure 1-30) with bacteria such as MRSA and *C. difficile* becoming household names (Figure 1-30). This was mainly caused by the overuse and inappropriate use of antimicrobial drugs (Fridkin 2001; Gould 1999; Talbot *et al*. 2006; Davies *et al*. 2013; O'Neill 2014). This is illustrated by the use of antimicrobials for *prophylactic* purposes in humans, livestock and agriculture (Bax

et al. 2000; Lipsitch 2002). Many animals have been administered antibiotics as growth promoters and consequently cross-resistance can occur with human antibiotics. In the last fifteen years, commonly used veterinary antibiotics such as avoparacin have been removed from veterinary formularies, this was due to its close similarity to vancomycin, an essential antibiotic in the treatment of MRSA (Hope *et al*. 2013; McGeer and Low 2000). The overuse of antibiotics has caused the strong positive selection of mutants and therefore the emergence of drug resistant bacteria (Aminov and Mackie 2007; McGeer and Low 2000).

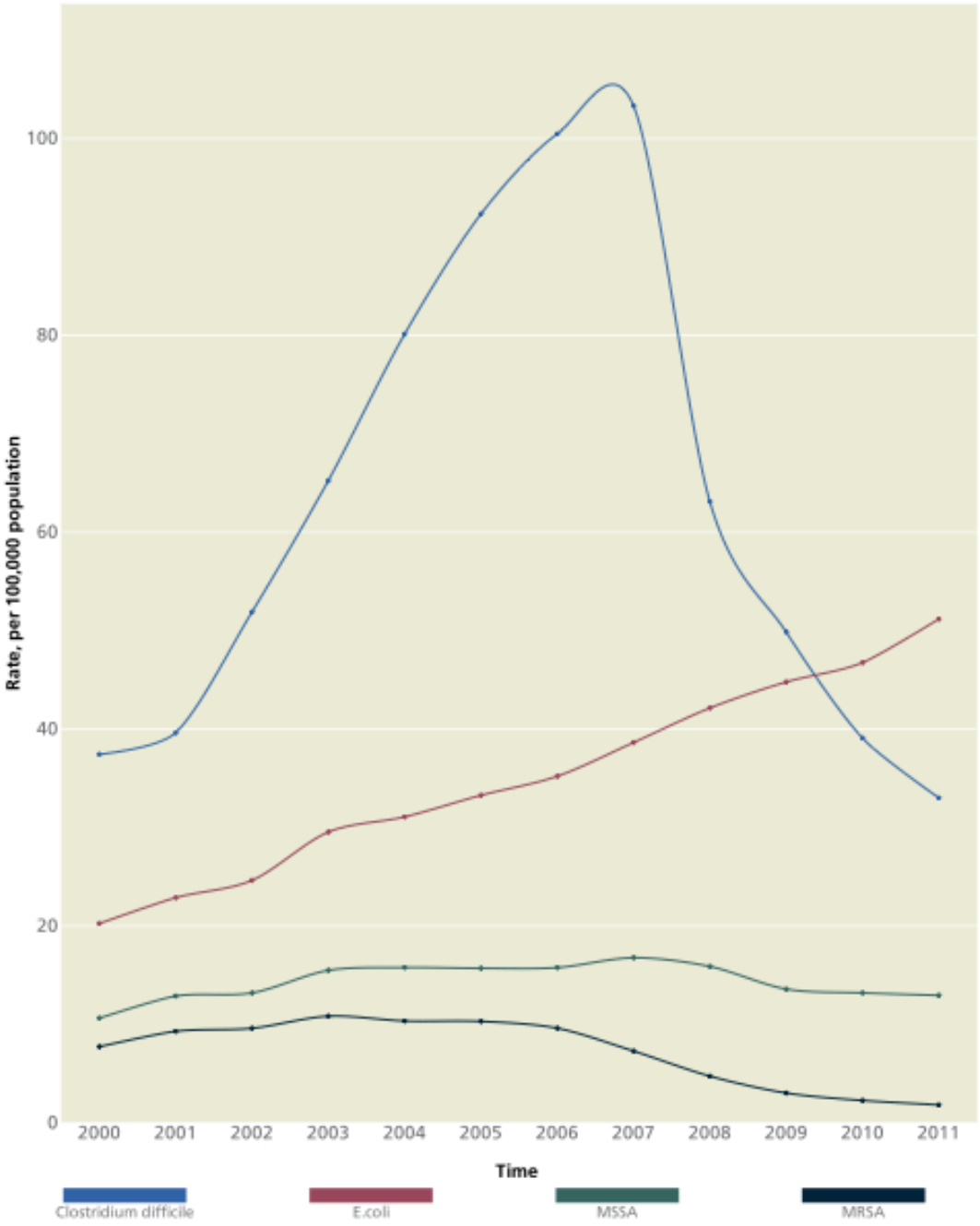


Figure 1-30 Trends in human *C. difficile* infection, MRSA, MSSA and *E. coli* bacteraemias, England, 2000 to 2010. Taken from (Davies *et al.* 2013)

The occurrence of resistant bacteria such as MRSA and *C. difficile* has decreased in recent years mainly due to policy changes and more stringent controls on essential antibiotics. Nonetheless, new challenges are materialising such as the dramatic increase in the number of patients contracting drug resistant *enterobacteriaceae* such as *E. coli* and *Klebsiella* (Davies *et al.* 2013)(Figure 1-31).

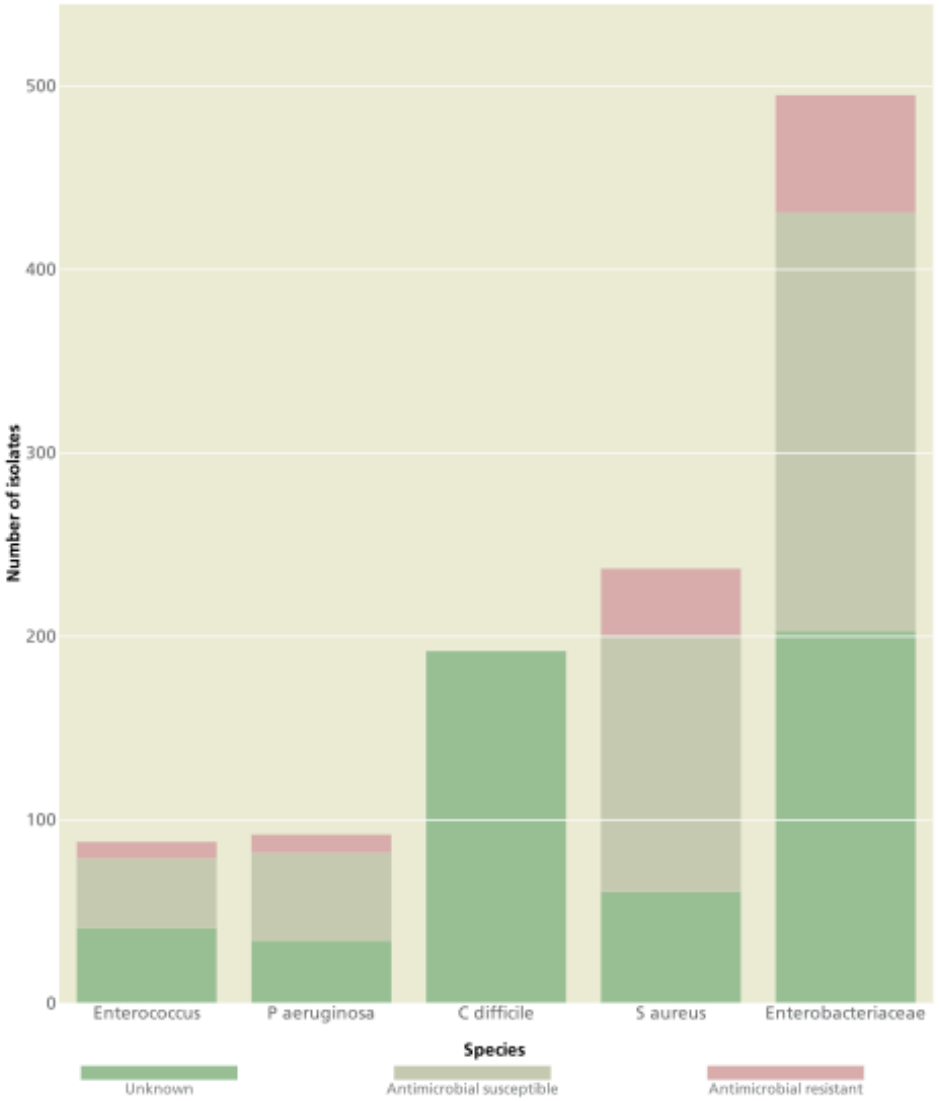


Figure 1-31 Leading pathogens in hospital patients by antimicrobial resistance, England 2011 taken from Davies *et al* (2013).

Patients in an intensive care unit (ICU) for over seven days are two- to three-fold more likely to come into contact with a resistant microorganism. This is mainly due to their immunocompromised status and their bodies lack of ability to resist infection (Fridkin 2001). This is a notable problem with MRSA (Dunlap 2007), a much maligned bacterium, due to its rise to prominence in the early 2000s (Figure 1-30), that is still very much in the public eye. This is a bacterium named after its resistance to methicillin, a beta-lactam antibiotic. A similar resistance profile is observed in related antibiotic agents such as flucloxacillin and benzylpenicillin, which are commonly prescribed for non-resistant staphylococcus aureus infections. This resistant strain has proved to be a major problem and although at the moment, in majority of cases, MRSA infection is still treatable, prevention of initial infection

with MRSA is still one of the top priorities among most hospital trusts (Dunlap 2007; Davies *et al*. 2013). *C. difficile* is another serious infection, often affecting the elderly, which is a consequence of increasing broad-spectrum antibiotic use. *C. difficile* is a serious threat to patients and to stop total resistance occurring, or at least to delay it, the use of broad spectrum antibiotics must be reduced (Fowler *et al*. 2007). Although MRSA and *C. difficile* are on the decrease (Figure 1-30), other Gram-negative infections such as *E. coli* and *Klebsiella* are on the rise. Gram-negative infections have increased by over two thirds in recent years and are now the most common cause of a hospital acquired infection (HAI) (Davies *et al*. 2013) and blood stream infections (Figure 1-32). With resistant infections common and other infections increasing, treatments are proving inadequate and new antimicrobials to replace the failing ones are desperately needed (Fowler *et al*. 2007).

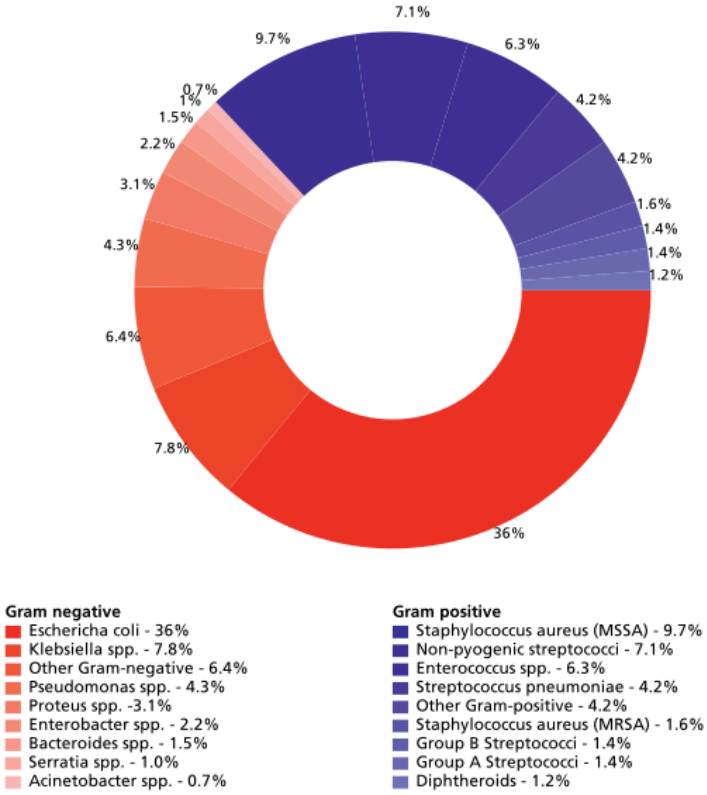


Figure 1-32 Organisms causing blood stream infections in adults in England, Wales and Northern Ireland April 11- March 12 taken from Davies *et al*. (2013).

1.5.6 The discovery void

The development of antibacterials started with the accidental discovery of penicillin by Fleming in the 1920s (Fleming 1929) and was followed in the 1930s and 1940s with

sulphonamides and other penicillins. In the following decades there was a continual steady production of new antibacterial drugs and most infections were well managed as drug companies had been consistently able to develop new drugs to act against resistant strains (Silver 2011) (Figure 1-33).

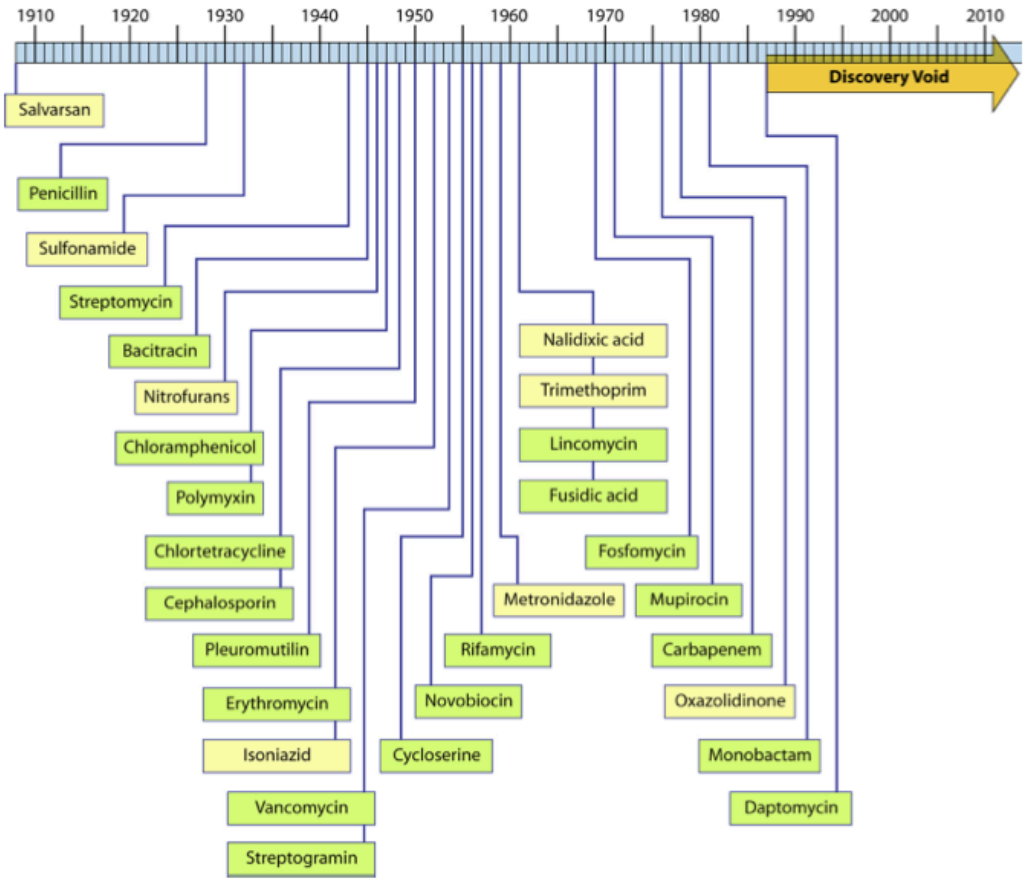


Figure 1-33 Diagram highlighting the discovery void. Dates shown are of initial discovery or patent taken from (Silver 2011).

Figure 1-33 highlights the previous successful development of a wide range of antimicrobial drugs, which have been effective in treating infections, but also emphasises the discovery void over the past 20 years. One of the reasons for this slow down and lack of recent development of new antibacterials, is that large drug companies do not see profit in research and production. This is because antibacterials are not frequently used for long periods of time; this reduces the amount of profit a pharmaceutical company can make from developing a solution. An antibacterial drug is normally used short term (when needed, for one or two weeks) and the profit margin is incomparable to that achieved for developing a treatment for a chronic condition such as diabetes or high blood pressure, where a patient would have to take the medication everyday for the rest of their life (Davies *et al*. 2013). This

lack of development of new antibacterials has added to the increased development of resistant organisms and may not only lead to therapeutic failure, but potentially to the death of many patients (Norrby *et al*. 2005; Davies *et al*. 2013).

1.5.7 Addressing the problem of drug-resistant infections

Current treatment for drug-resistant infections includes the use of drugs such as vancomycin (Hope *et al*. 2013), which have poor toxicity profiles in comparison to other treatments such as beta-lactams, where resistance is now problematic (Swartz 1994). The current antibacterials used against resistant bacteria are often drugs once rejected for their toxicity profile, but have been 'brought back' to combat resistance. Not only do drugs such as vancomycin have unpleasant side effects, but resistance to them is starting to become more common. An example includes the new strains known as vancomycin-resistant *enterococci* (VRE) (Davies *et al*. 2013). The best way to slow down this growth in resistance is to reduce the amount of antibiotics we use and save our best antibiotics for essential use (Davies *et al*. 2013). This will however only delay the process, as resistance may not only occur not just from over-use of antibiotics, it can also arise through natural bacterial evolution. These essential drugs will still be used, just less frequently, only delaying the appearance of resistant microorganisms. While slowing the increase of resistant strains down is very important and we must continue to do this, we will need to find new anti-infective agents to combat resistance in the future. The annual report of the chief medical officer targeted seven key areas of focus as part of a strategy for combating antimicrobial resistance (listed below) (Davies *et al*. 2013). Behavioural changes and improved infection prevention make up two of the key areas of the list and the 5th area identified is facilitated development of new antimicrobials. The Department of Health (DoH) have identified that themselves, the pharmaceutical industry and the EU, are all essential to the development of novel antimicrobials (Davies *et al*. 2013).

Seven key areas of focus were highlighted as part UK antimicrobial resistance strategy (Davies *et al*. 2013).

1. Promote evidence based prescribing
2. Improve infection prevention control
3. Raise public and professional awareness of antimicrobial resistance threat and promote behaviour change

4. Research programme into new diagnostics, alternatives to antibiotics (such as antiseptics), pathogenesis, effective behavioural change to improve infection prevention and control and prescribing practice
5. **Facilitate the development of new antimicrobials and vaccines**
6. Improve surveillance and data linkage
7. Encourage international collaboration and date sharing and learning from best practice internationally

With little current research, and resistance escalating, one possible approach of identifying novel antimicrobials would be to study organisms that have already resolved such problems through evolution. All macro-organisms, whether it's the simple marine sponge, or a large mammal, possess an innate ability to resist infection from the wide variety of microorganisms within their habitat (Evans *et al*. 1997). In the past 35 years, 73% of all antibacterial drugs approved were derived from natural sources and were made up of only three classes of drug: beta-lactams, macrolides and quinolones (Hughes and Fenical 2010). New avenues of research, such as antimicrobial discovery from the sea (Lawrence 2015), may be able to find new solutions to the resistance problem, rather than for example analogues of current medicines, which can be more easily overcome. This is particularly important when looking for new mechanisms of action which nature may have already resolved. By understanding how various organisms combat microorganisms in their natural environment, we might be able to discover new antimicrobial drug structures and utilize the knowledge gained in this search to aid the fight against drug-resistant infections

1.6 Aim and objectives

The overall aim of this PhD was to screen marine sponges as targets for novel drug-like molecules. The objectives of this project were to:

- Identify and prioritise unstudied marine sponges from unexplored environments.
- Develop efficient methods to extract compounds from marine sponges.
- Screen a large number of marine sponges for novel, bioactive, compounds extracted directly from marine sponge material and develop methods to characterise and dereplicate them with limited starting material.
- Cultivate, identify and investigate the bioactivity of bacteria isolated from marine sponges.
- Compare the chemical profile of marine sponges and the associated bacteria that colonise them.

Chapter 2
Materials and methods

2 Materials and methods

2.1 Materials

2.1.1 Reagents and solvents

All general laboratory reagents and solvents were purchased from Sigma Aldrich (Poole, UK) or Fisher Scientific (Loughborough, UK) and were of analytical standard or molecular biology grade unless otherwise stated. High performance Liquid chromatography (HPLC) grade solvents were used for spectroscopic techniques such as mass spectrometry (MS) and counter current chromatography (CCC).

2.1.2 Equipment and glassware

All general laboratory equipment and glassware was purchased from Sigma Aldrich (Poole, UK) or Fisher Scientific (Loughborough, UK).

Equipment	Model	Manufacturer
Pipettes	P20N, P200N, P2000N	Gilson, UK
Analytical balance	AB54-5	Fisher, UK
Desktop balance	B303-S	Mettler-Toledo
Centrifuge	Biofuge Primo R	Thermo scientific, UK
Centrifuge	Coulter Avanti J-20 XP	Beckman Coulter Inc, USA
Microscope	BX50	Olympus, UK
Rotavap	Rotavapor R-124	Büchi, Germany
Magnetic stirrer	VB15001	Fisher, UK
UV lamp	UVGL-55 254/365 nm	UVP, Canada

2.2 Sponge samples

2.2.1 Sponge delivery and storage

2.2.1.1 Greek samples

All Greek samples were sun dried in Greece before delivery to the UK.

2.2.1.2 Welsh samples

Welsh samples were collected by hand of by using self-contained underwater breathing apparatus (SCUBA) at varying depths. Upon collection they were cryopreserved at −80 °C with no preparation (samples intended for chemical extraction) or suspended in 10 % v/v DMSO or 30 % v/v glycerol (in artificial sea water or the sea water the sample was collected in), to protect any bacteria present, prior to cryopreservation. Samples for chemical extraction were freeze-dried (Lyolab 3000 6L, Thermo Scientific Heto, UK) for 24 h.

2.2.2 Processing sponge for solvent extraction

Each sponge sample had inherently different physical properties with some samples retaining elasticity upon freeze-drying. For this reason a method for processing each sample into a uniform powder was developed. Each sponge sample was sliced down the middle of all visible oscula using a sterile scalpel, and any visual contamination (*e.g.* polystyrene balls/plastics/other invertebrate species) was removed with sterile tweezers. The sample was then cut into small pieces ~1 cm^2 and ground in a pestle and mortar to form a fine powder. If a sponge sample was not of sufficient brittleness for grinding, it was flash frozen in liquid nitrogen to decrease its elasticity and aid processing. The weight of the finely ground material was recorded before extraction.

2.2.3 Polar extraction

Processed sponge samples were extracted using solvents of increasing polarity over a period of two days. Three consecutive extractions were completed using hexane-acetone-methanol (a low to medium to high polarity gradient). A suspension was prepared in an Erlenmeyer flask, to a concentration of 2% (w/v) and stirred for 24 h at room temperature, using a magnetic stirrer (VB15001, Fisher, UK) to a create a vortex. After 24 h the suspension was filtered using vacuum filtration and Whatman® grade 1 filtration paper with the resulting solid being refreshed with the subsequent solvent. Extractions were conducted over a period of 48 h. If the solution appeared saturated, the solvent was refreshed after 24 h giving two solutions for each solvent used, which were combined and then dried to a solid using a rotary evaporator (Rotavapor R-124, Büchi, Germany) and weighed, prior to storage at 2 – 8 °C.

This extraction method produced three dried extracts from each sponge (with increasing polarity), an initial mass (allowing yield to be calculated) and a final mass of un-extracted solid residue allowing calculation of insensible losses.

2.2.3.1 Soxhlet extraction

Soxhlet extraction was also employed for some samples, post methanol extraction. It was decided not to utilise this method for all samples as it produced little increase yield and there was a decomposition risk for any desired products due to the temperatures involved.

2.3 Chemical analysis of sponge extracts

2.3.1 Analytical thin layer chromatography (TLC)

Analytical chromatography was performed using aluminium backed TLC plates (20×20cm silica gel $60F_{254}$, Merck Kieselgel, Germany). Each sample analysed was spotted onto a labelled baseline (marked with pencil 1 cm from the base of the plate) using a glass tube or a pipette to dispense exactly 10 µL of sample for controlled experiments. A concentration of 20 mg mL^{-1} was used when the dry weight of the sample was known. If the concentration was unknown, the sample was visualised under UV light before separation to ensure the concentration of the sample was sufficient for visualisation when separated. Multiple compounds were tested in various solvent systems to ensure efficient separation of each sample to allow direct comparison of their constituents.

The loaded plates were removed following elution and the solvent front level was marked. Analytical TLC plates were visualised under short wave UV light (254 nm), long wave light (365 nm), and with iodine vapour staining and vanillin spray. The retardation or retention factor (Rf) value of each separated compound was calculated using Equation 2-1.

$$Rf = \frac{distance\ travelled\ by\ compound}{distance\ travelled\ by\ solvent\ front}$$

Equation 2-1 Calculation of the retention factor (Rf) adapted from (Sherma and Fried 2003).

2.3.2 TLC purity tests and prevention of rediscovery

Purity tests of compounds were completed using analytical TLC by spotting the compound for testing and the authentic sample on top of each other and next to each other. If when eluted no separation was noted and the single spots appeared the same, the compounds were considered to be identical.

2.3.3 Ultraviolet (UV) visualisation

UV light was used as a simple method of visualising any compounds that were UV active, notably compounds with extensive conjugation such as benzene rings. Eluted TLC plates were allowed to dry at room temperature and then placed under a UV lamp (UVGL-58 handheld lamp, UVP, Canada). Visible spots were circled lightly, with a pencil, upon visualisation under the lamp before being removed unaltered. Visualisation was completed at UV wavelengths of 254 and/or 365 nm (Figure 2-1). UV light provides a fully reversible method of visualisation and was the primary method used to visualise compounds on TLC plates unless otherwise stated.

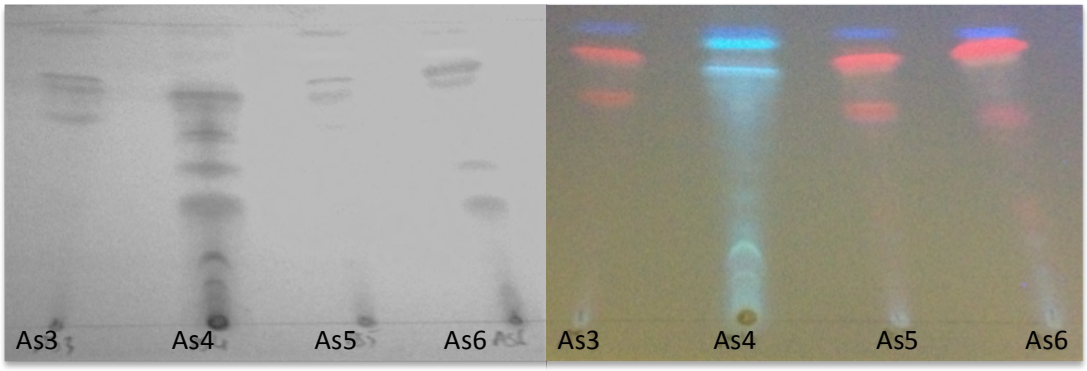


Figure 2-1 Digital image of examples of UV visualisation of TLC plates (4:6 acetone : hexane) separated from Greek sponge acetone extracts (labelled): As3, As5 and As6 (*Chondrilla nucula*) and As4 (*Petrosia ficiformis*). (A): UV 254 nm, (B): UV 365 nm.

2.3.4 Vanillin spray visualisation

Vanillin spray (vanillin 15 g, ethanol 250 mL, concentrated sulphuric acid 2.5 mL (Wall 2005)) was sprayed onto eluted analytical TLC plates to saturate the surface. Plates were laid horizontally to prevent the solution running down the plate. Once saturated, the plates were warmed with a heat gun (Steinel HL500, Steinel, Germany) until a colour change was visible.

Plates were then allowed to cool and visual images were recorded using a digital camera (Figure 2-2). Vanillin staining was used as a non-reversible method of visualisation, where compounds could not be recovered from a plate post-staining.

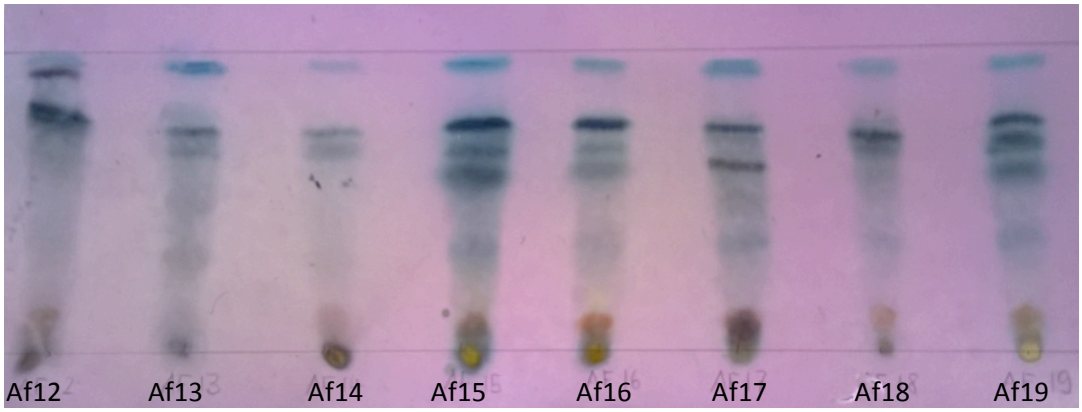


Figure 2-2 Digital image of an example of vanillin stain visualisation of TLC plates (4:6 acetone : hexane) using Greek sponge acetone extracts (labelled): Af12 and Af18 (*Sarcotragus sp.),* Af14 (*Agelas oroides*), Af15 and Af16 (*Crambe crambe*) and Af17 (*Petrosia ficiformis*).

2.3.5 Iodine staining

Iodine staining was used as a fully reversible stain with a strong affinity for both aromatic and unsaturated functional groups, leaving compounds embedded onto a TLC plate unaltered. Iodine crystals were placed into the bottom of a sealed glass chamber, which subsequently saturates with iodine vapour as iodine sublimes. An eluted TLC plate was then inserted and the chamber re-sealed until a light brown colour develops evenly over the entire plate. Compounds with high affinity for iodine stained dark brown (Figure 2-3). The plate was then carefully removed and any dark brown areas were quickly circled with a pencil, and Rfs recorded before the iodine evaporated (Wall 2005).

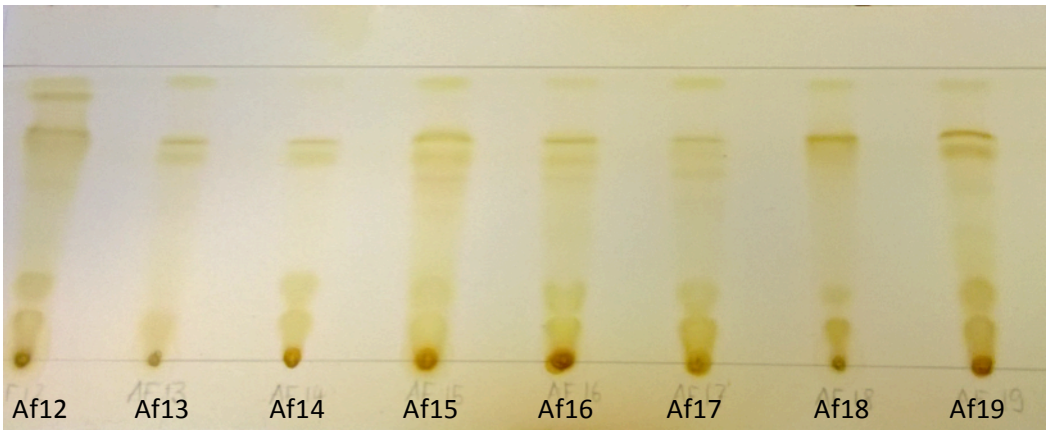


Figure 2-3 Digital image of an example of iodine stain visualisation of TLC plates (4:6 acetone hexane) using Greek sponge acetone extracts: Af12 and Af18 (*Sarcotragus sp.*), Af14 (*Agelas oroides*), Af15 and Af16 (*Crambe crambe*) and Af17 (*Petrosia ficiformis*).

2.3.6 Preparative TLC

After finding a suitable solvent system using analytical TLC (section 2.3.1), preparative TLC was employed to separate compounds on a larger scale. 100 mL of the same solvent system developed with analytical TLC, was decanted into the bottom of a larger TLC tank. Up to 100 mg of sample was then re-suspended, in as little solvent as possible, and slowly pipetted onto a pencil line 1 cm from the base of a glass backed 1 mm silica plate (20 × 20 cm $60F_{254}$, Merck Kieselgel, Germany). Samples ranging between 100 – 200 mg of crude material were separated on 2 mm plates (20 × 20 cm $60F_{254}$, Merck Kieselgel, Germany). It was important to allow the TLC plates to dry between sample applications; as a solution applied on top of a saturated area would start to partially elute during sample loading. After elution, the solvent front was recorded to allow calculation of Rf.

Completed chromatograms were then visualised by UV (254 and/or 365 nm) depending on the wavelength the fractions were visible at when analytical TLC was used. Iodine vapour staining was used to detect spots that were not visible under UV. A pencil was used to mark visible spots, which were then scraped off using a spatula. Each individual band was stored separately, mixed with solvent and labelled with the furthest eluting fraction as 1. For example, the furthest eluting compound from the hexane extract (lowest polarity) of *Cliona celata* would be 1CC1 (Figure 2-4).

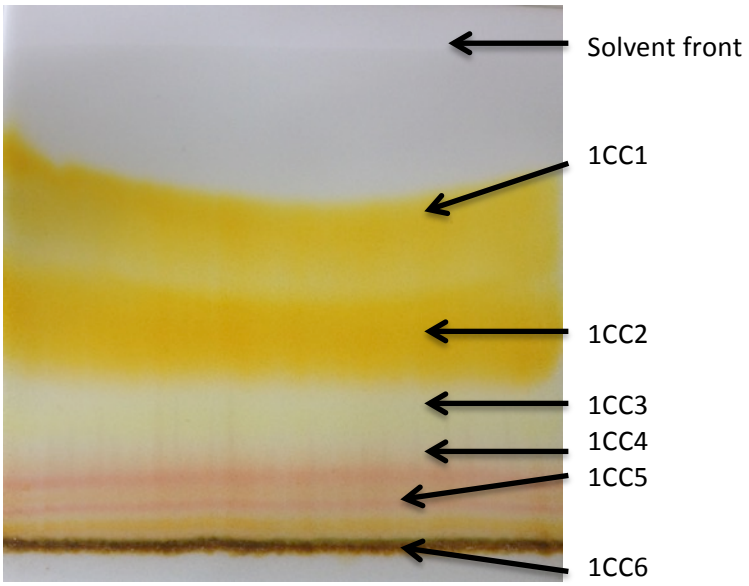


Figure 2-4 Digital image of a preparative TLC separation (1 mm silica plate, 20 × 20cm 60F_{254}, Merck Kieselgel, Germany) of the hexane extract of Cliona celata (CC) collected from Wales (2:8 hexane : acetone).

Each suspension was then stirred for 24 h in an attempt to extract as much of the sample as possible from the silica (Figure 2-5). All suspensions were then filtered using a syphon filter apparatus and the resulting solutions were dried using rotary evaporation. The resulting solids were weighed and tested using analytical TLC for purity.

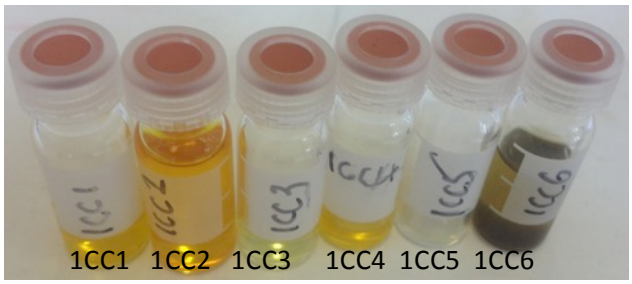


Figure 2-5 Digital image of fractions formed post separation using preparative TLC of the hexane extract of Cliona celata (CC) collected from Wales, fractions suspended in hexane (labelled as shown in Figure 2-4).

2.3.7 Column Chromatography

Two types of column chromatography were used to separate larger quantities of extracts.

2.3.7.1 Silica column chromatography

A silica column was used to separate larger quantities of compounds (> 200 mg), in place of preparative TLC. Analytical TLC was used to find a suitable solvent system (mobile phase)

with gradient elution used during the elution to push more polar molecules through. The column was prepared using the least polar solvent system, mixing it with silica and suspending it in the column. The silica was then allowed to settle and form a level surface for the stationary phase.

Solid or liquid extracts were dissolved in a minimum volume of solvent, and slowly pipetted onto the surface of the saturated stationary phase. The extract solution was then allowed to elute until it had been fully absorbed into the stationary phase. The solvent system was then pipetted on top of this, to allow the column to run. Care was taken to ensure the column was constantly oversaturated as drying the column could alter the chromatography kinetics.

As the column was running, test tubes were used to collect the fractions of solution produced. Each test tube was checked by visualisation on a TLC plate under UV light. The polarity of the solvent system was gradually increased throughout the process to allow even separation of compounds from a sample.

Once all the material had passed through the column, fractions collected were tested using analytical TLC. Fractions with matching or similar profiles were then combined and dried. If any sample was still impure, the chromatography process could be repeated.

2.3.7.2 Size exclusion chromatography

A Sephadex LH-20 (GE Healthcare, UK) column was established to separate extracts based on the size of the molecule instead of its polarity. Size exclusion chromatography was used as an efficient way of not only separating the compounds but also to limit the size of any compounds encountered at an early stage.

The column was set up using the instructions provided (GE Healthcare, UK) and the process was evaluated using known molecules, as standards, to find conditions with a good spread with clear distinction between large molecular weight (MW) (> 500), medium (200 - 500) and small (< 200) molecules. Various solvent systems were tested by ensuring the column was rested for at least 18 h and settled between changes. After each run the column was washed through with methanol. Following assembly of the column and stationary phase the same separation method was followed as for a Silica column from (2.3.7.1).

2.3.8 Efficient separation process

All chromatography techniques were used to achieve the most efficient separation possible. The following flow diagram (Figure 2-6) was employed to ensure the correct flow of techniques was used.

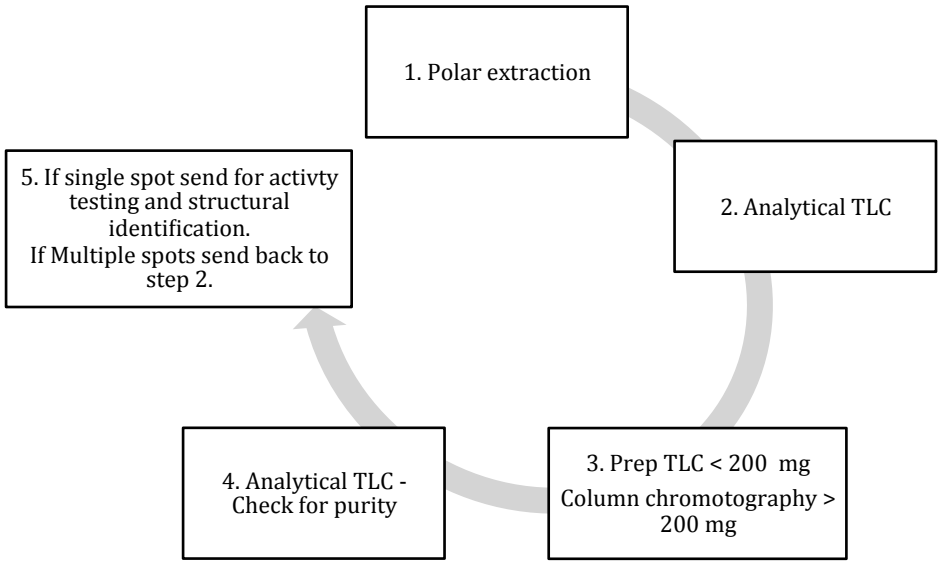


Figure 2-6 Separation flow diagram.

2.4 Spectroscopic analysis of isolated compounds

2.4.1 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance was used as a method of spectroscopy to aid the determination of the structure of a molecule as well as its purity. Protons (^{1}H) or carbon-13 atoms (^{13}C) can be excited by radiofrequency radiation and become aligned with rather than against a magnetic field. The structure of a molecule can be elucidated from the range of frequencies needed to excite the atoms and the splitting patterns produced. NMR chemical shifts were reported in reference to the peak of the solvent and signal splitting patterns were recorded as singlet (s), doublet (d), triplet (t) and multiplet (m). All isolates were suspended in dimethyl sulfoxide-d6 (DMSO-d6) unless otherwise stated. All samples were analysed by ^{1}H NMR and/or ^{13}C NMR using a Bruker Avance DPX500 spectrometer operating at 500 MHz and 125 MHz, respectively.

2.4.2 Mass spectrometry (MS)

Mass spectrometry was used as an analytical method to acquire information about a compound's molecular mass. Molecules are ionised by the removal of one or more electrons. The resultant ions are then accelerated through an electric or magnetic field onto a detector. This process separates molecules by mass allowing determination of their molecular weights.

Preliminary MS was performed using electrospray (ES) MS providing low-resolution mass spectrometry using a Bruker microTOF spectrometer. Low-resolution analysis allowed the exclusion of known molecules and those with high molecular weights and provided a reference mass for high resolution MS.

Promising lead compounds (appropriate MW and biological activity), were sent to the School of Chemistry (Cardiff University) or the EPSRC National Mass Spectrometry service (Swansea University) for high resolution electron impact (EI) MS in positive and negative mode. This analysis provided accurate mass and breakdown products for more detailed information about the chemical composition of the molecule and its possible molecular formula.

2.5 Microbiological methodology

2.5.1 Aseptic methods

Where necessary, materials were sterilised by autoclaving at 121 °C for 15 min. Aseptic techniques were performed as required and carried out in a class 2 biosafety cabinet with 70 % (v/v) ethanol in water used as the general disinfectant.

2.5.2 Microbiological media and diluents

2.5.2.1 Media

Mueller-Hinton agar (MHA) and broth (MHB) and nutrient agar (NA) and broth (NB) were purchased from Thermo Fisher Scientific Ltd, UK.

Actinomycete isolation agar (AIA) and Phosphate buffered saline (PBS) were purchased from Sigma Aldrich Ltd., UK. Marine Broth and Agar (2216) were from BD Difco™, USA.

All media were prepared according to the manufacturer's instructions and sterilised before use.

2.5.2.2 Diluents

Deionised water (diH_2O) was decanted from an ELGA Purelab Option BP15 dispenser (ELGA labwater, UK) and sterilised before use.

2.5.3 Bacterial cultures

Methicillin sensitive *Staphylococcus aureus* (MSSA) (NCIMB 9518), *Escherichia coli* (*E. coli*) (NCIMB 12210) and methicillin-resistant *Staphylococcus aureus* (MRSA) (NCTC 11939) were obtained from Cardiff University's culture collection. MSSA and *E. coli* were chosen for initial screening, as they represent examples of Gram-positive and Gram-negative bacteria, respectively. All manipulations with these bacteria were completed in a class 2 biosafety cabinet.

A bacterial culture was subcultured by filling a 50 mL centrifuge tube with 30 mL of NB and adding the bacteria from the stock culture using an inoculation loop. The resultant broth was incubated for 16 h at 37 °C with shaking at 100 rpm (Microspec, UK). The broth was then centrifuged at 5000 rpm for 15 min at 18 °C (Coulter Avanti J-20 XP, Beckman Coulter Inc, US). The supernatant was removed and the pellet was re-suspended in fresh NB or PBS and diluted to the desired optical density for the chosen test.

2.5.4 Surface viable count – the Miles Misra method

A surface viable count was completed for each test bacterium to determine the number of colony forming units (CFU) found in a bacterial suspension (Miles *et al*. 1938). This was completed as follows-

2.5.4.1 Method

- A culture of the chosen bacterium was grown over 16 h in NB. This suspension was then centrifuged at 2600 ×g for 15 min until a pellet was formed. The resulting supernatant was then removed and the broth was refreshed with fresh sterile PBS and the suspension was then thoroughly dispersed using a vortex.

- The resultant suspension was then serially diluted (1:10) eight times to 10^{-8}. Each dilution was run in triplicate to ensure the sample size was large enough to ascertain statistical significance.
- NA plates were divided into eight equal segments, which were labelled with the increasing dilutions.
- On each marked segment, 10 µl bacteria suspension (of the appropriate dilution) was dropped three times and allowed to spread and dry naturally with no overlap between drops.
- The plates were then left facing upwards to dry before they were inverted and incubated at 37 °C in an incubator (Memmert Ltd, UK) for 24 h.
- Each segment was then visually inspected for growth. High concentrations of the bacterium gave confluent growth over the drop area and colonies were counted on the segment with the largest number of clear discrete colonies (2-20 colonies).

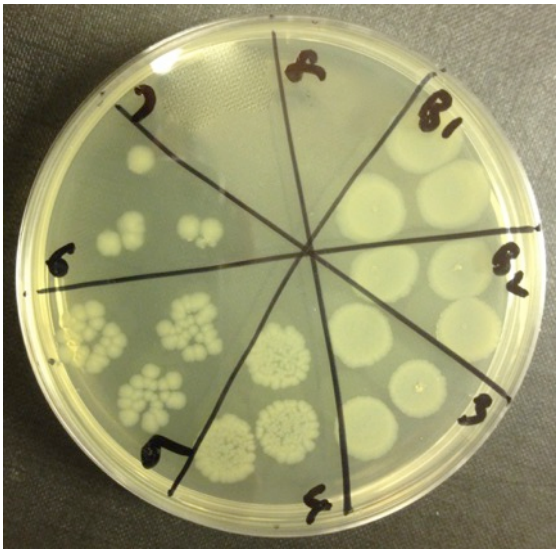


Figure 2-7 An example of a surface viable count of *E. coli*. The segment labelled 6 contains three drops with six distinct colonies making the average number of colonies for this plate two with a dilution factor of six (ten fold).

Colony forming units per mL of the original sample was calculated using the following equation.

$CFU\ mL^{-1}$ = Average number of colonies for a dilution × 100 × dilution factor

2.5.4.2 Determination of the concentration of bacterial cultures by the construction of an OD_{600} vs Bacterial $CFUmL^{-1}$ graph

Sterile nutrient broth was inoculated with either *E. coli,* MSSA or MRSA and then incubated overnight in a shaking incubator programmed at 100 rpm and 37 °C. After 16 h the culture was removed from the incubator and centrifuged at 3000 ×g for 15 min (Coulter Avanti J-20 XP, Beckman Coulter Inc., USA). The supernatant was then removed and discarded and the pellet was re-suspended in 15 mL of PBS to provide the neat dilution. This suspension was then serially diluted and the optical densities were measured and recorded at 600 nm using a spectrophotometer (Amersham Biosciences Ultrospec 3100). The Miles Misra method (section 2.5.4) was used to record a viable count for the construction of an OD_{600} *vs* $CFUmL^{-1}$ graph. Figure 2-8 shows the data for *E. coli,* providing the linear equation $y = 5 \times 10^{-9}x$ allowing the prediction of the number of bacteria present in a sample based on its optical density. Using this formula, it was estimated that at 600 nm, 1×10^{8} bacteria were present in an optical density (OD) of 0.5. MSSA and MRSA had similar profiles whereby an OD of 0.5 was indicative of 1×10^{8} bacteria present.

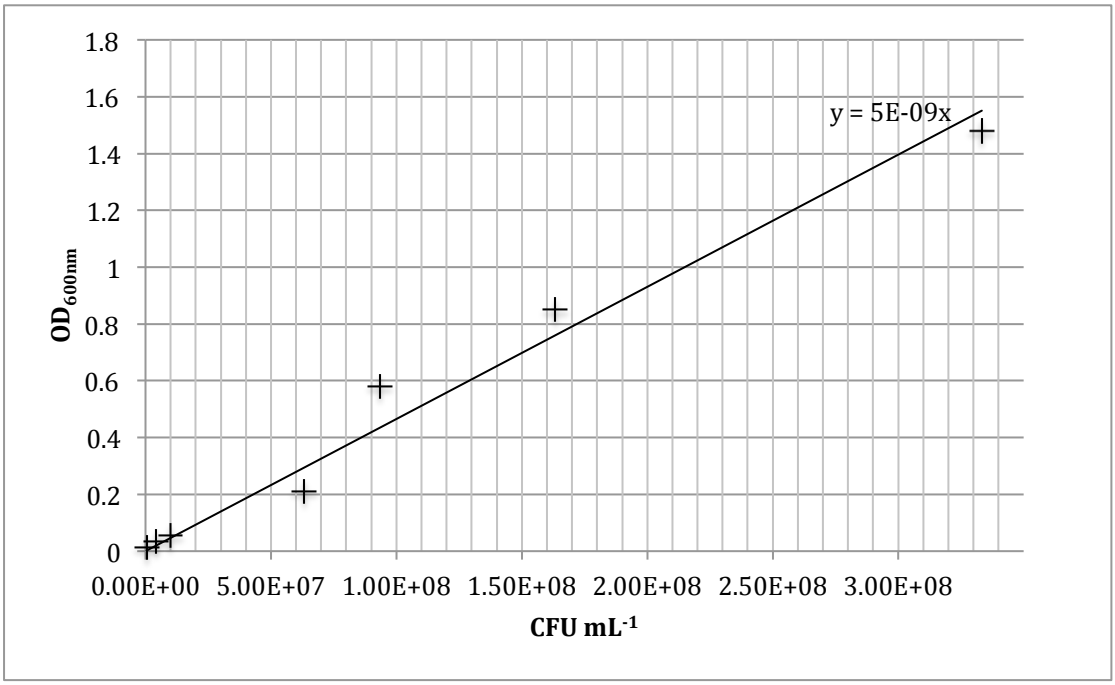


Figure 2-8 An example of OD_{600} *vs* CFU mL^{-1} graph of *E. coli*.

2.5.5 Obtaining single colonies by plate streaking

Isolates/colonies obtained from bacterial cultivation were removed using a sterile inoculation loop and spread in a small area of a sterile NA plate. A new sterile inoculation loop was then used to streak the plate in straight lines in a circular motion (Figure 2-9). Streaked plates were then incubated at 37 °C for 18 h, plates were then visually inspected for the isolation of single colonies, if resolution of colonies had not been achieved the process was repeated.

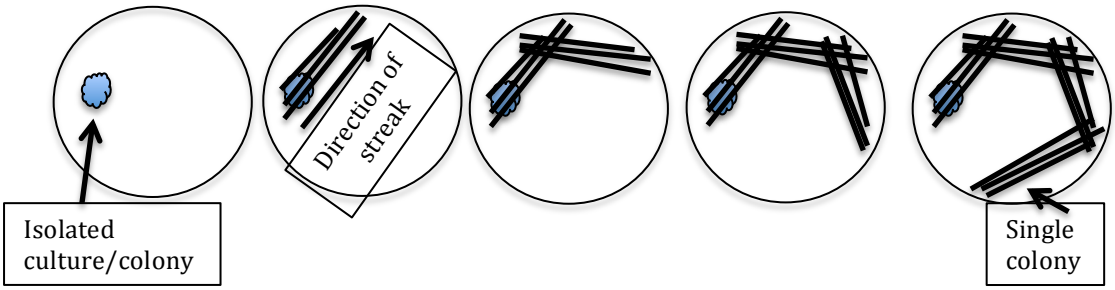


Figure 2-9 A schematic representation of streaking an agar plate.

2.5.6 Storage of bacteria by the preparation of fresh bacterial culture slopes

All three types of bacteria (MSSA, MRSA and *E. coli)* used in this study were kept on bacterial culture slopes, which were stored at 2-8 ^{o}C and replaced every two weeks from freezer stocks. Slopes were prepared by adding 10 mL of sterile molten NA to a sterile McCartney bottle (20 mL) and allowed to set at an angle of 30^{o}, thus producing a gradient slope of agar. The chosen bacterium was thawed from a freezer stock at room temperature and then aseptically inoculated onto the slope using a sterile loop. The inoculated slope was then incubated overnight at 37 ^{o}C and the resulting culture was tested for purity by the plate streaking method (Figure 2-9).

2.5.7 Staining procedure

2.5.7.1 Heat fixing

Bacteria were prepared for staining by heat fixing to slides using the following steps:

1. Using a sterile pipette tip, 50 µL of sterile diH_2O was pipetted onto the centre of the slide.

2. Using a sterile 10 µL inoculating loop, a single colony was transferred to the diH_2O and mixed to create a turbid suspension.
3. The turbid solution was then spread across the slide to produce a translucent thin film or smear.
4. The prepared slide was then left to dry next to a Bunsen burner and using tweezers, the slide was rapidly passed through the Bunsen flame in order to heat fix the bacteria. The heated slide was then allowed to cool.

2.5.7.2 Gram Stain

Gram staining of microbes was completed to visualise and aid identification of microbial species under a microscope (Halebian *et al*. 1981) using the following steps:

1. A heat fixed slide (section 2.5.7.1) of the chosen bacterium was incubated at room temperature with 2 % w/v crystal violet dissolved in 20 % v/v ethanol in diH_2O solution for 1 min.
2. Excess crystal violet solution was then poured of the slide, which was then incubated at room temperature with Gram's iodine (Pro-Lab Diagnostics, UK) for 1 min.
3. The slide was then rinsed with diH_2O to wash away excess iodine, washed with 50% v/v acetone in ethanol for 15 s before being rinsed with diH_2O.
4. The slide was left to incubate at room temperature with 2.5 % w/v Safranin dissolved in 95 % w/v ethanol in diH_2O as a counter stain for 10-15 s before being rinsed with diH_2O to removed excess safranin.
5. The stained fixed bacteria were then left to air dry.
6. Slides were then viewed under a microscope to a total magnification of ×1000 (BX50, Olympus, UK).

2.6 Statistical analysis

Wherever possible statistical analysis was performed within this study to confirm the significance of any results obtained. All results presented represent the mean of three results unless otherwise described. All data and statistics were analysed using IBM® SPSS® Statistics version 20 for Mac and the choice of test used followed the flow diagram in Figure 2-10**.**

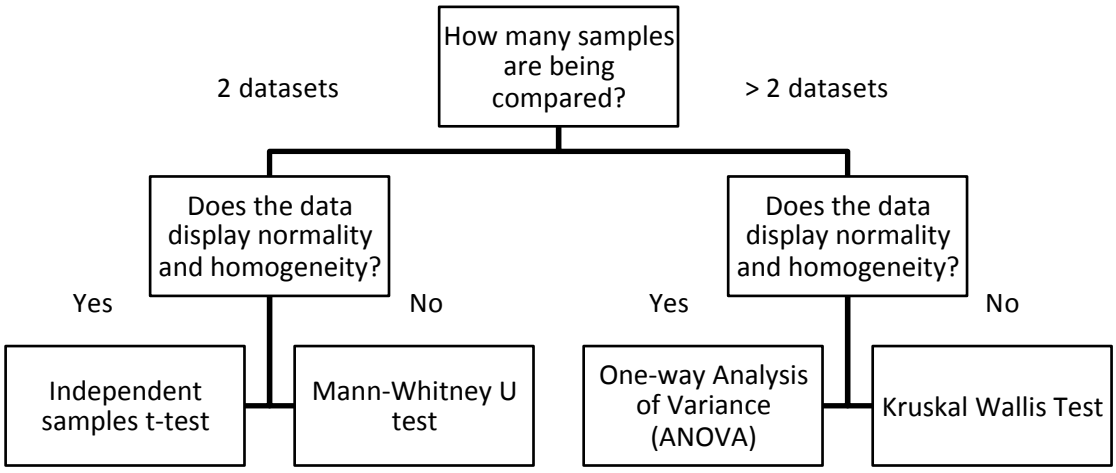


Figure 2-10 Flow diagram for choice of statistical method of analysis.

2.6.1 Comparison of two variables

2.6.1.1 Independent samples t-test

The parametric independent Student's t-test was used to determine statistical difference in the means of two groups if the results were independent of one another, the results followed normal distribution and the data sets showed homogeneity of variance (Cohen 1988). Normality was assessed using a histogram plot to determine if a normal bell shaped curve was observed with minimal kurtosis and skewedness. These results were used in conjunction with Kolmogorov-Smirnov and Shapiro-Wilk tests of normality. Levine's test was also completed to determine if the data showed homogeneity of variance. If these assumptions were not met the data was transformed in an attempt to normalise the data *e.g.* natural logarithm, square root, $\log_{10}$ etc.

The effect size between the two groups was calculated with Equation 2-2:

(A) $$\eta^2 = \frac{t^2}{t^2+(n1+n2-2)}$$

(B)

Effect size	η^2
Small	0.10 - 0.29
Moderate	0.30 – 0.49
Large	> 0.50

Equation 2-2 (A) Calculation of the magnitude of difference between two groups or the eta-squared value η^2. *t* = *t* value from t-test, *n*1 = sample size of group 1 and *n*2 = sample size of group 2. (B) Interpretation of the effect value defined by (Cohen 1988).

2.6.1.2 Mann-Whitney U Test

This non-parametric test was employed to compare the difference in two groups if the assumptions made for the independent samples t-test were not met. If a significant difference was observed the effect size was calculated with

(A) $$r = \frac{z}{\sqrt{n}}$$

(B)

Effect size	*r*
Small	0.10 - 0.29
Moderate	0.30 – 0.49
Large	> 0.50

Equation 2-3 (A) Calculation of the magnitude of difference between two non-parametric groups. r = effect size, z = z value generated from test and *n* = total sample size. (B) Interpretation of the effect value defined by (Cohen 1988).

2.6.2 Comparison of more than two variables

2.6.2.1 One-way Analysis of Variance (ANOVA)

If the assumptions described in 2.6.1.1 were met the parametric ANOVA test was employed to ascertain if the difference between more than two groups of data was significant. If the results of the ANOVA test found a significant difference between groups, Tukey's multiple comparison test was employed to establish which groups were different from one another.

2.6.2.2 Kruskal Wallis Test

This was the most commonly employed test throughout this project as most datasets produced a non-parametric result due to the low number of repeats (three) for the majority of samples. This test is essentially the non-parametric equivalent of one-way ANOVA describing if the medians of each group were significantly different. The Kruskal Wallis test was employed for datasets of greater than two when the assumptions outlined in 2.6.1.1 were **not** met.

Chapter 3
Collection, extraction and chemical analysis of Greek sponges

3 Collection, extraction and chemical analysis of Greek sponges

3.1 Chapter introduction

Marine sponges contain a complex mix of compounds, many of which are active against a variety of different microbes and cancer cell lines (Ankisetty and Slattery 2012). This mixture of compounds, of varying polarity, can be extracted using a variety of different solvents from low to high polarity, with like dissolving like.

When investigating the chemical constituents of marine sponges most researchers complete their first extraction with methanol or a mix of methanol and dichloromethane (Galeano *et al*. 2011; Ankisetty and Slattery 2012). The disadvantage of this method is that some non-polar compounds may be instantly lost as they will not be extracted. In this thesis, a prime objective was to develop efficient methods to extract compounds from marine sponges. Therefore, a step-wise extraction process was utilised starting with non-polar extraction. While this method is recognised for extraction of marine natural products (Houssen and Jaspars 2012) a search of comparable research projects found it is rarely employed for sponge samples. A similar method has been previously employed for sponge extraction (Tsoukatou *et al*. 2002) but this method was not sequential and simply completed three extracts from fresh sponge material. Another study completed a step-wise extraction with acetone and then methanol but combined the samples and then fractionated using a separating funnel (Kumar *et al*. 2012). The main advantage of the stepwise extraction employed in this study, over the above methods, is the instant separation into three different fractions with no loss of material.

Recent sponge research has focused on the concept that bacteria are the true producers of the active secondary metabolites isolated from marine sponges and has targeted the associated bacteria found on sponges for novel compounds (Debbab *et al*. 2010; Anand *et al*. 2006; Viegelmann *et al*. 2014). The research in this thesis involved the characterisation of antibacterial compounds extracted directly from a sponge sample followed by a comparison to those extracted from cultures grown from the host sponge.

Extraction was first attempted with two well-characterised species, *Agelas oroides* and *Aplysina aerophoba*, as a proof of concept for the proposed method of extraction. Novel compounds could still be extracted as no sponges have ever been chemically characterised from the surrounding Aegean Sea around Samos and differences in water temperature and location are known to affect sponge metabolite production (Sacristán-Soriano *et al*. 2011; Sacristán-Soriano *et al*. 2012).

3.1.1 Agelas oroides

Agelas oroides (*A. oroides*) is taxonomically classified as follows-

Animalia (kingdom) > Porifera (phylum) > Demospongiae (class) > Agelasida (order) > Agelasidae (family) > Agelas (Genus) (Van Soest 2007).

A. oroides is only found in seawater and is distributed in the following locations: Adriatic Sea, Aegean Sea, Croatia, Croatian Exclusive Economic Zone, European waters (ERMS scope), Greek Exclusive Economic Zone, Levantine Sea, Mediterranean Sea, Monaco and the Western Mediterranean (Braekman *et al*. 1992).

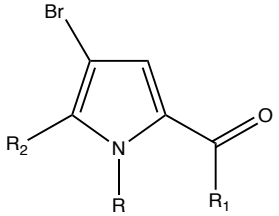
The sample studied was found in the Aegean Sea in the nets of local fisherman (Figure 3-1). Like many other warm water sponges, *A. oroides* has been widely studied and multiple agents with antibacterial and anticancer activity have already been extracted (Ferretti *et al*. 2007; Ferretti *et al*. 2009; Cachet *et al*. 2008; Forenza *et al*. 1971).

(A) **(B)**

Figure 3-1 Digital images of *Agelas oroides* samples. (A) *Agelas oroides in situ* (Archipelagos Institute of Marine Conservation, taken on September 7, 2011, available from: http://www.flickr.com/photos/archipelago-flickr/6148977053/**, accessed March 2015). (B) Dried specimen found in the waters around Samos.**

The Agelas genus has been well studied however a search on MarineLit (March 2015) identified only 13 'new' compounds isolated from the species *Agelas oroides*. The genus and

species *A. oroides* are known to contain bromopyrrole alkaloids (Figure 3-2, Figure 3-3), which are also common to Axinellidae and Halichondria sponges (section 1.4.3). Oroidin (Figure 3-3), which is considered the precursor of the bromopyrrole alkaloids, is named as such due to its discovery from extraction of *A. oroides.*



Number	R	R_1	R_2
1	H	OH	Br
2	H	OMe	Br
3	H	CN	Br
4	H	NH_2	Br

Figure 3-2 Bromopyrrole alkaloids found in *Agelas oroides*.

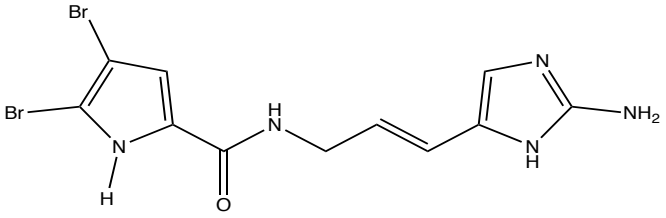


Figure 3-3 Oroidin, a bromopyrrole alkaloid isolated from *Agelas oroides.*

Further compounds identified on MarinLit include non-brominated pyrroles and the more complex antibacterial agelorins, cycloroidin and the antihistaminic taurodispacamide A (Konig and Wright 1993; Fattorusso and Taglialatela-Scafati 2000).

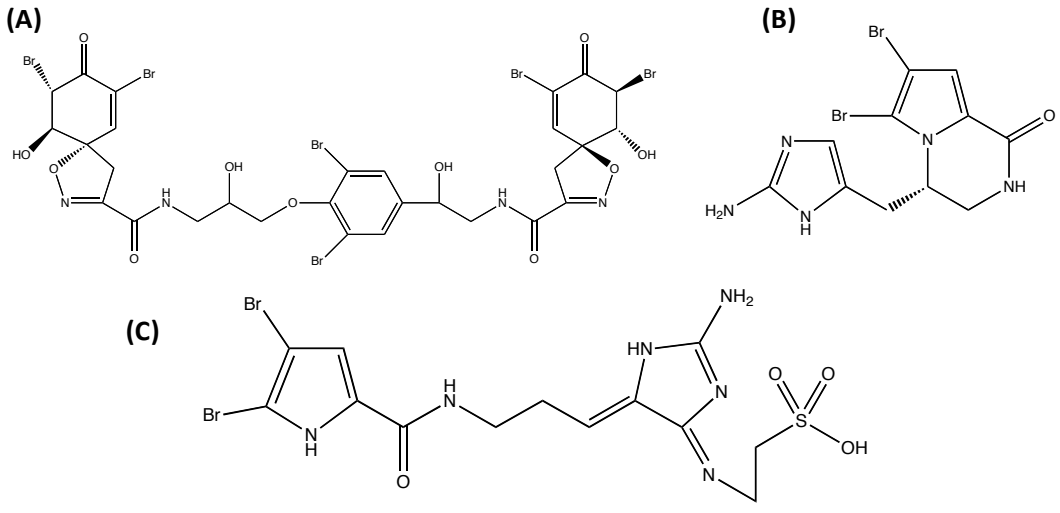


Figure 3-4 Structure of (A), Agelorin A; (B), Cycloroidin and (C), Taurodispacamide A.

3.1.2 Aplysina aerophoba

Aplysina aerophoba (*A. aerophoba*) is bright yellow sponge found predominantly in and around the Mediterranean Sea (Fattorusso *et al*. 1970). It was notable upon collection that on removal from the seawater, it almost instantly lost its bright yellow colour hence the name *aerophoba*; this could be due to the oxidation of pigments on the exposure to air.

A. aerophoba is taxonomically classified as follows-

Animalia (kingdom) > Porifera (phylum) > Demospongiae (class) > Verongida (order) > Aplysinidae (family) > Aplysina (Genus) (Van Soest 2007).

It is a member of the *Verongida* order of sponges, which are known to be responsible for the production of hundreds of bromotyrosine alkaloids (Aiello *et al*. 1995). Narrowed down further, *A. aerophoba* is part of the *Aplysinidae* family, which along with *Aplysinellidae*, *Ianthellidae*, and *Pseudoceratinidae* are responsible for the production of over 90% of bromotyrosine alkaloids (Aiello *et al*. 1995). Bromotyrosine alkaloids are known to possess a diverse range of medically relevant bioactivities including antibacterial (D. Kim et al. 1999), antiparasitic (Galeano *et al*. 2011), anticancer (Tran *et al*. 2013) and anti-HIV activity (Ross *et al*. 2000). Up to 10% of the dry weight of *A. aerophoba* is made up from the brominated isoxazoline alkaloids. Examples of the compounds previously isolated are aplysinamisin-1, aerophobin-2 and isofistularin-3, which upon damage to the sponge tissue are bioconverted by enzyme catalysed cleavage to the monocyclic nitrogenous compounds aeroplysin-1 and its related dienone (isoxazoline precursors (Figure 3-5)). Aeroplysinin-1 and its related dienone have shown cytotoxic and antibacterial activities, partly through the formation of free radicals (Thoms *et al*. 2006).

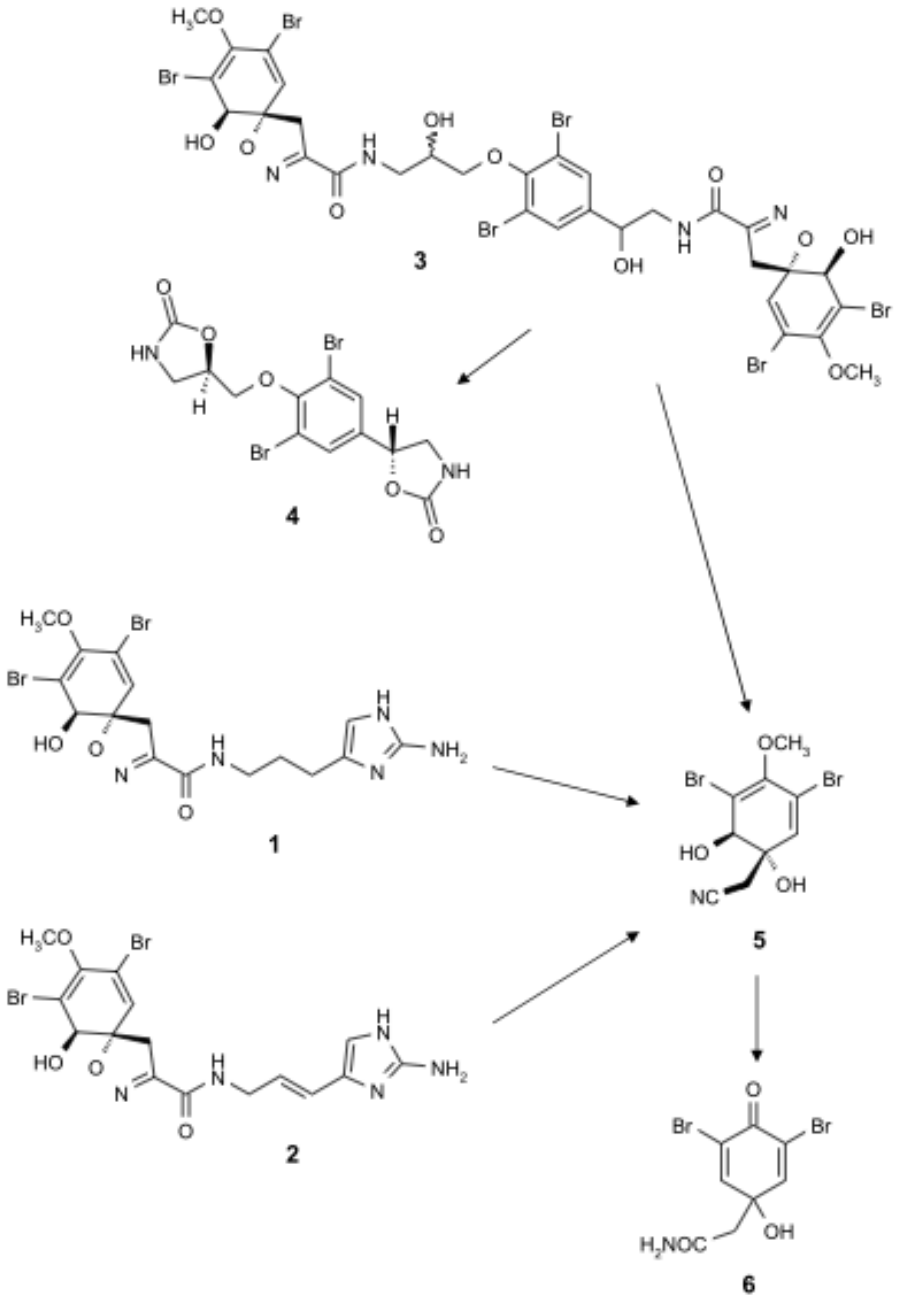


Figure 3-5 Schematic of wound-induced bioconversion of the brominated isoxazoline alkaloids aerophobin-2 (1), aplysinamisin-1 (2), and isofistularin-3 (3) to aeroplysinin-1 (5) and the dienone (6) in tissue of *Aplysina aerophoba*. When isofistularin-3 (3) is used as a substrate for the reaction, the bisoxazolidinone derivative (4) is recovered as a further product. Taken from Thoms *et al.* (2006).

MarinLit (searched March 2015) identified 24 compounds isolated from *A. aerophoba* and similar to *A. oroides* this seems relatively low to what may be expected. There is some variation in products with unusual sterols (aplysterol), aplysinadiene and an oxazolidinone (Norte *et al*. 1988), however many of the other compounds found in MarinLit are actually

results from sponge associated fungus and bacteria (Pimentel-Elardo *et al*. 2010; Gernot Brauers *et al*. 2000).

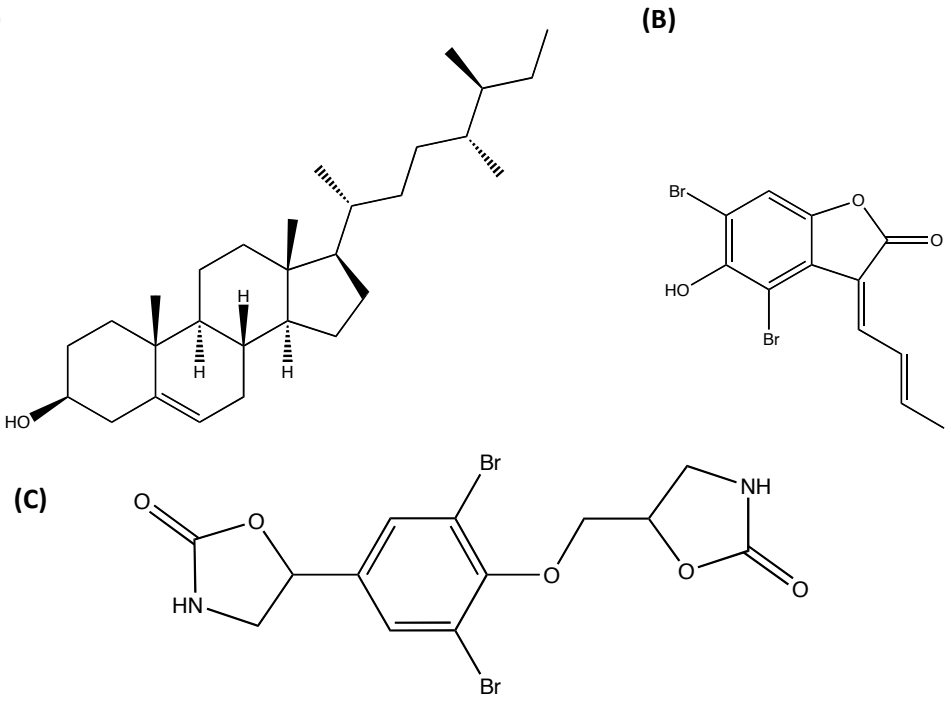


Figure 3-6 Structure of (A), Aplysterol; (B), Aplysinadiene and (C), an oxazolidinone specific to *Aplysina aerophoba*.

3.1.3 Identification of 'drug like' compounds from marine sponge extracts - Lipinski's rule of five (Lipinski *et al*. 1997)

Lipinski's rule of five is a retrospective list of physiochemical characteristics a compound requires to produce good oral bioavailability. Conformity with these criteria gives an indication that a molecule could be considered as a 'good drug'. The rules are as follows:

- Molecular weight (MW) of < 500
- LogP of < 5
- < 5 hydrogen donors
- < 10 hydrogen acceptors

By no means is this an exhaustible list and there are many exceptions to these rules. In fact some particularly undesirable compounds, *e.g.* strychnine (Statham 1956), can be considered drug-like by meeting the criteria, and potentially better compounds may be discarded for missing the criteria of one of the rules, *E.g.* vancomycin (MW 1449, 21 hydrogen bond donors and 33 acceptors) is not orally bioavailable but is effective when giving orally for the treatment of *Clostridium difficile* due to a direct action in the colon

(Korman 2015). Antibiotics are described by Lipinski as one of the exceptions to these rules with large molecules such as erythromycin (Figure 1-27) still bioavailable most likely due to their derivation from nature. However treatment regimes are now starting to rely on drugs such as gentamicin and vancomycin, which have to be given systemically to achieve activity. It has to be considered the list developed by Lipinski was a retrospective study looking at trends and not a guarantee of what make a good drug. These historic large orally bioavailable antibiotics, that have been successful clinical drugs (*E.g*. macrolides), will have influenced the results and prompted the exemption from Lipinski's criteria however it is more likely for a drug to be bioavailable and make it through clinical trials if it possesses a lower molecular weight.

In this research study, molecules were limited by their molecular weight. Any compounds over MW 550 were not considered further for the following reasons:

- A significant number of lead compounds were likely to be isolated during the course of this study and the numbers had to be limited for purely practical reasons.
- Smaller molecules are easier to identify and structurally characterise with the techniques and resources available. Marinomycin A, which was isolated from *Marinispora* took two years to structural characterise and realistically that timeframe and resource demand was outside the scope of this PhD (Kwon *et al*. 2006).
- The molecules included are more likely to be useable if developed further due to good bioavailability by fitting the criteria identified by Lipinski (1997).

While this limit was regularly applied throughout this study, exceptions were considered if the MW of a compound was less than 600, if clear antibacterial activity was identified and there were no obvious matches from known compounds.

3.2 Chapter aim and objectives

The primary aim of this chapter was to establish techniques for the isolation and identification of active compounds from marine sponges using limited starting material. The objectives of this chapter were to:

- Successfully extract and separate compounds from a sponge mass.
- Assess the antibacterial activity of sponge extracts.
- Characterise as completely as possible compounds that cause activity, which are novel and drug-like.
- Discover the limitations of separation and identification techniques and develop a method to screen a large number of sponges.

3.3 Methods

The first extractions and isolations were completed on two sponge species that are well characterised allowing the opportunity to predict and compare likely isolates, such as the bromopyrrole and bromotyrosine alkaloids (Figure 3-2, Figure 3-3, Figure 3-5).

3.3.1 Pre-collected Greek samples

Samples of *A. oroides* and *A. aerophoba* were collected from the Aegean Sea surrounding the Archipelagos research station on the Island of Samos, Greece (Figure 3-7).

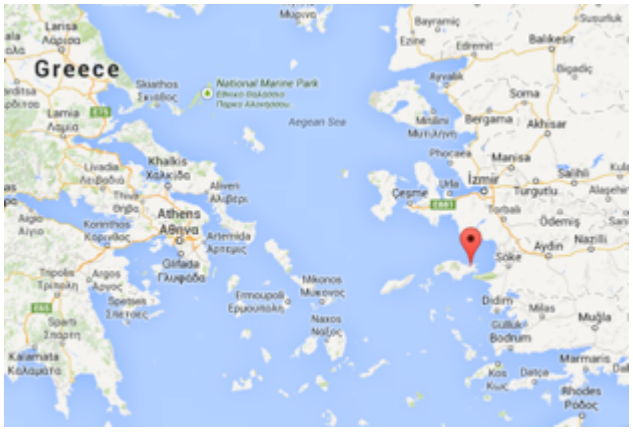


Figure 3-7 A digital image of a map depicting the geographical location of Samos, Greece, which is near the Western coast of Turkey. ('Samos, Greece.' *Google Maps*, Google. 2015, available from : https://www.google.com/maps/place/Samos,+Greece/@38.1213118,25.7194684,8z/data=!4m2!3m1!1s0x14bc3f9fd1d780f5:0x4ba91c89f2bbdc7f?hl=en-US (accessed March 2015).

Samples were collected from unknown depths after being found in nets of local fisherman and dried by sunlight. In the laboratory, they were stored at room temperature.

3.3.2 Solvent extraction and chemical analysis of sponge material

Sponge samples were processed (section 2.2.2) and extracted using the sequential solvent extraction method (section 2.2.3) forming three extracts for each sample. Chemical analysis was completed for the resultant extracts (section 2.3) followed by spectroscopic analysis of isolates (section 2.4).

3.3.3 Soxhlet extraction

This method uses a very small volume of solvent due to its re-circulation system, driven by the siphon on the side. It is more effective than methanol extraction alone as it is heat driven; which is also a disadvantage as thermo-labile compounds may be degraded (Bucar *et al*. 2013).

Soxhlet extraction was completed for *A. aerophoba* with the solid obtained by methanol extraction. Soxhlet extraction was used as a method that allowed the extraction of chemicals from a solid leaving behind insoluble impurities (Hawthorne *et al*. 2000). The method employed is listed in the following steps:

1. The sample was placed is a paper thimble shaped container in the centre of the apparatus (A, Figure 3-8).
2. The reservoir at the bottom, containing the solvent, was heated to reflux using an oil bath to maintain temperature (B, Figure 3-8).
3. Upon reflux, the solvent rises and condenses into the sample in a steady drip (C, Figure 3-8), dissolving the desired compounds from the solid.
4. As the solvent fills the reservoir, it also rises in the syphon, the syphon then flushes drawing the solvent and the solubilised compounds into the bottom reservoir (D, Figure 3-8), this process was cyclical and continued for 48 h.

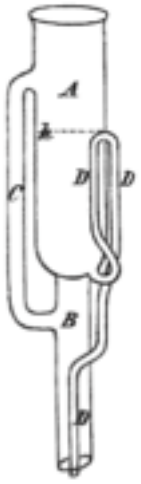


Figure 3-8 Soxhlet's original drawing of his extractor. (A) Central reservoir containing paper thimble and dry sample, condenser positioned above, causing volatilised solvent to slowly drip into sample; (B) Solvent positioned in heated beaker below; (C) Solvent evaporates and rises through column before condensing into central reservoir; (D) Syphon allowing recirculation of solvent (W. B. Jensen 2007).

3.3.4 Measuring zones of inhibition: Well method

Müller-Hinton agar (MHA) was prepared as per manufacturer's instructions and transferred to a laminar flow cabinet where 20 mL aliquots were transferred into sterile petri dishes (90mm). After the agar had cooled and set, plates were inoculated with 200 µL of pre-prepared 10^5 CFU mL^{-1} of MSSA (NCIMB 9518) suspended in PBS (2.5.3), which was spread evenly to produce a lawn growth, using a sterile spreader.

A 6 mm borer was sterilised using ethanol and flaming, before cutting holes into the agar producing four wells per plate, the borer was re-sterilised, and cooled, between each well. All extracts were dried and suspended in DMSO to 10 mg mL^{-1}. DMSO was also used as negative control and vancomycin (10mg mL^{-1}) as a positive control. 25 µL of each suspension was pipetted into the wells using a fresh sterile tip each time, completing each experiment in triplicate. Plates were incubated for 16 h at 37 °C.

Zones of inhibition were calculated by measuring the diameters of the clear zones with no growth (Figure 3-9). These diameters where averaged and the 6mm size of the well was subtracted (Equation 3-1).

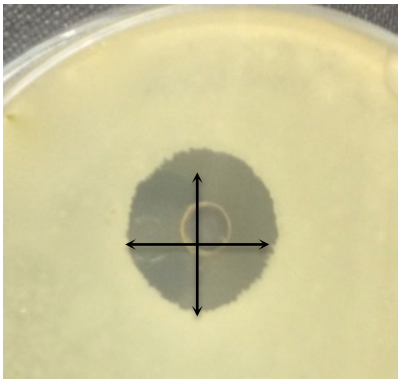


Figure 3-9 Digital image of activity observed against MSSA using well method. Average area of inhibition calculated by taken two measurements (orthogonal to one another) across the clear area of inhibition (indicated by arrows).

$$Zone\ of\ inhibition = Average\ diameter\ of\ inhibition - Diameter\ of\ well$$

Equation 3-1 Calculation of zone of inhibition.

3.4 Results and discussion

3.4.1 Method development: Size exclusion chromatography

All Sephadex column experiments were completed as described in section 2.3.7.2. Sephadex swells differently in different solvents, and this impacts on separation. Therefore, some preliminary tests were completed to achieve best separation of extracts. A Sephadex column was assembled using 100 g of Sephadex in a glass column, which was prepared to allow separation of compounds via their molecular weight rather than their polarity (Carr *et al*. 1971). This column however proved too small to provide good separation, as compounds did not have enough distance to separate, hence a 200 g column was assembled instead. Three compounds, with a variety of molecular weights were put into solution and tested for efficient separation through the column (Figure 3-10).

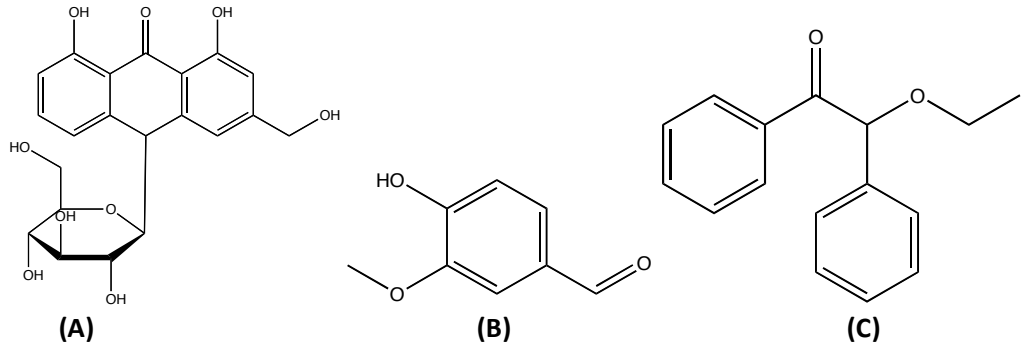


Figure 3-10 (A) Structure of Aloin MW 418. (B) Structure of Vanillin MW 152. (C) Structure of Benzoin ethyl ether MW 240.

Multiple solvent systems were trialled and the key results were as follows:

1. Methanol caused the Sephadex to swell to almost double the size of hexane, but allowed solids to run through as one and provided poor separation. Methanol was notably excellent for cleaning column.
2. 10:10:80 (hexane : methanol : acetone) provided good separation of compounds when tested with standards. This solvent combination also provided a good separation of acetone sponge extract. It provided a poor separation of non-polar (hexane) extracts with all products eluting at once. This may be due to similarity in mass but also possibly due to the polarity of the solvent system. Increasing hexane and decreasing methanol ratios for non-polar extracts solved this problem.

3. High concentrations of chloroform and dichloromethane caused the Sephadex to lift off the bottom of the column. For this reason, no more than 25% of either solvents were included as part of a solvent system to keep Sephadex at the bottom of the column.

3.4.2 Isolation and identification of the chemical constituents of *Agelas oroides (Ao)*

3.4.2.1 Preliminary extraction of Agelas oroides

A. oroides (20 g) was ground to a fine powder (section 2.2.2) and extracted over 48 h intervals with solvents of increasing polarity (hexane, acetone and methanol), producing three extracts (Figure 3-11) (section 2.2.3). Samples were named based on the abbreviation of sponge species and proceeded by a number, which represented the solvent used for extraction, hexane (1), acetone (2) and methanol (3). For example, the methanol extract of *A. oroides* was named 3Ao. Any further chromatographic separations where numbered after the species abbreviation. *E.g.* 3Ao1 and 3Ao2 for two separated components of the methanol extract of *A. oroides.*

Figure 3-11 A digital image of *Agelas oroides* extracts in round bottom flasks. (hexane extract (1Ao), acetone extract (2Ao), methanol extract (3Ao))

Initial analysis, via analytical TLC, (section 2.3.1) of each extract showed a variety of compounds present with some similarities between each fraction. Similar spots on the TLC plates across each extract showed that although stirred in solvent for 48 h, extraction was incomplete. In total 5.446 g of material was extracted giving a 27 % yield from dry sponge. As expected, most material (4.954 g) was extracted in the methanol extract (3Ao), which produced a very complex TLC analysis with poor resolution of bands. The acetone extract (2Ao) however, produced a manageable quantity of product, 0.174g, which could be separated using a preparative TLC plate. The acetone extract also showed very clear

distinctive spots on analytical TLC in 2:8 (acetone : hexane). The fractions were separated using preparative TLC in 2:8 (acetone : hexane) (Table 3-1).

Table 3-1 Rf values and observed appearance of fractions of the acetone extract of *A. oroides* (2Ao). Extract separated by preparative TLC using 2:8 (acetone : hexane).

Compound	Rf	Appearance
2Ao1	0.8	Thin colourless band visible under UV (254nm)
2Ao2	0.5	Thin colourless band visible under UV (254nm)
2Ao3	0.3	Wide orange band visible under UV (254nm)

Upon extraction from the silica the newly formed fractions were again analysed using TLC. 2Ao1 in 1:19 (acetone : hexane), 2Ao2 in 2:8 (acetone : hexane), 2Ao3 in 6:4 (acetone : hexane).

After elution 2Ao2 produced two distinct bands when visualised with UV light (254nm). It was separated further on 1 mm preparative TLC plate with 2:8 (acetone : hexane). 2Ao1 was also separated further using preparative TLC 1:19 (acetone : hexane) to remove any potential impurities. Fractions 2Ao1, 2Ao2.1 and 2Ao2.2 were compared using analytical TLC in 2:8 (acetone : hexane) (Table 3-2).

Table 3-2 Rf values and observed appearance of fractions of the acetone extract of *A. oroides* (2Ao) visualised using analytical TLC 2:8 (acetone : hexane). The three fractions were formed following separation of fractions *2Ao1 and *2Ao2 using preparative TLC, (2Ao1 in 2:8 (acetone : hexane), 2Ao2 in 1:19 (acetone : hexane).

Compound	Rf	Appearance
2Ao1	0.8	Colourless single band visible under UV (254nm)
2Ao2.1	0.8	Colourless distinct band visible under UV (254nm)
2Ao2.2	0.5	Colourless distinct band visible under UV (254nm)

TLC purity tests (section 2.3.2) confirmed that fractions 2Ao1 and 2Ao2.2 were both pure samples but also likely to be the same compound as they eluted together upon TLC separation. To test this hypothesis further, more robust spectroscopic analysis was needed such as HPLC, MS and NMR. The ^{1}H NMR (Figure 3-12) confirmed 2Ao1 and 2Ao2.1 to be the same compound and it was likely a dialkyl phthalate ester (Figure 3-13, Figure 3-13), a commonly encountered plasticiser and almost certainly of human origin. The symmetry in these compounds led to a characteristic multiplet located in the aromatic region of a ^{1}H NMR spectrum, which represented the four aromatic hydrogen's. The additional peaks are typical

of branched chain alkaloids, which give a plasticiser its flexibility, further confirming the likelihood that these compounds were both dialkyl phthalate esters.

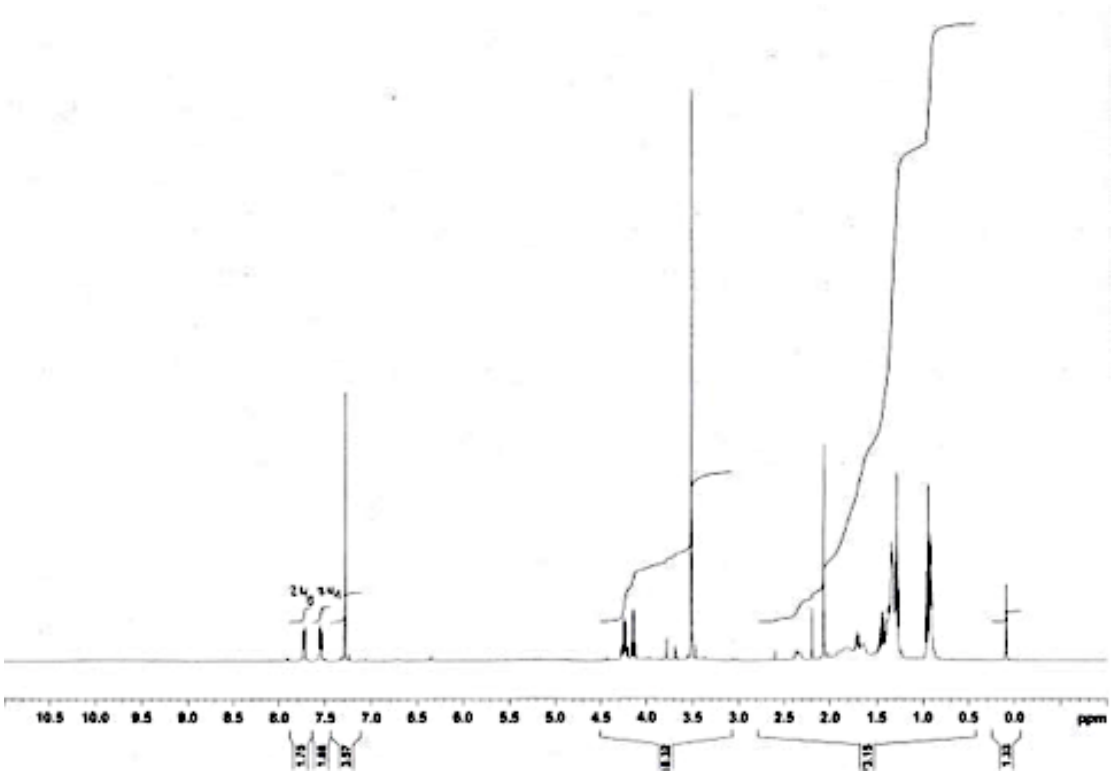


Figure 3-12 ^{1}H NMR spectrum of 2Ao1, a fraction of the acetone extract of *Agelas oroides* (2Ao) identified as dialkyl phthalate.

3.4.2.2 The origin of plasticiser contaminants

Dialkyl phthalate esters are the most commonly used plasticiser in the world and are an essential part of common plastics such as polyvinyl chloride and polyvinyl acetate (Mackintosh *et al*. 2004).

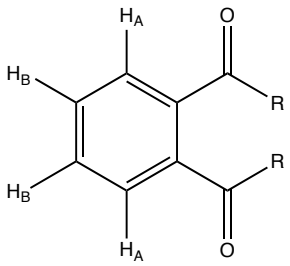


Figure 3-13 Basic chemical structure of Dialkyl phthalate ester.

Plastics can be easily mechanically broken down into micro-plastics that are prone to the leaching of components such as phthalate plasticisers, which may produce carcinogenic and

teratogenic effects (Teuten *et al*. 2009; Cole *et al*. 2011; Barnes *et al*. 2009). These plasticisers can be ingested by marine organisms, such as sponges, and invade tissues (Teuten *et al*. 2009). Plastics are very common on beaches in and around the Aegean Sea (Figure 3-14) and this may explain why these plasticisers are found in these sponge extractions.

Figure 3-14 Digital image of plastic litter on a beach in Samos.

Plasticisers could have been introduced into the sponge sample in two different ways:

1. They may have been introduced *in situ*, in the sponge's natural environment.
2. They may have been introduced post-removal from the natural habitat, either through carriage (storage in plastic bags) or as a contaminant in part of the separation process.

As the plasticisers may have been introduced in the laboratory it was decided that any further sponge samples would be more thoroughly inspected for foreign particulates to help reduce false leads. The plasticiser samples were also kept as standards for further isolations.

The remaining sponge sample was visually inspected and Figure 3-15 shows what looks to be man-made polystyrene balls, which were found in the sponge upon delivery. This potential contaminant was therefore introduced into the sponge in its natural environment and it was thought that the polystyrene balls could be the source of the man-made compounds found in the samples.

(A) **(B)**

Figure 3-15 Close up digital image of plastic contaminants in *Agelas oroides.* (A) Suspected polystyrene balls identified with arrows; (B) Plastic also found embedded in sponge.

Laboratory tests were conducted to eliminate the polystyrene balls as the potential source of the contaminant. The white spheres were firstly removed with tweezers and dissolved in acetone. Next the dissolved polystyrene was eluted against 2Ao1, 2Ao2.1 and 2Ao2.2 to see if they gave the same profile under the same conditions using analytical TLC 2:8 (acetone : hexane). When eluted with it was clear that it was not the polystyrene balls that had caused the appearance of plasticiser in the samples, as there were no visual similarities observed (2.3.2). It was apparent however that contaminants were a serious problem and it was decided that any polystyrene or other foreign objects would have to be carefully removed to reduce further contaminants in subsequent studies.

Further foreign particles (Figure 3-15) were identified in the sample of *A. oroides* reinforcing how thorough future processing of sponge had to be. Due to this risk, it was decided to repeat the extraction to get 'cleaner' extracts. Sponges were cut down each osculum (Figure 3-16) and any visual foreign particles were removed with tweezers. It was impossible to remove all foreign particles as many appeared embedded in the sponge and trapped in tiny pores, which was impossible to definitely distinguish from sponge material and remove.

Figure 3-16 A digital image of *Agelas oroides* cross-sectioned down an osculum.

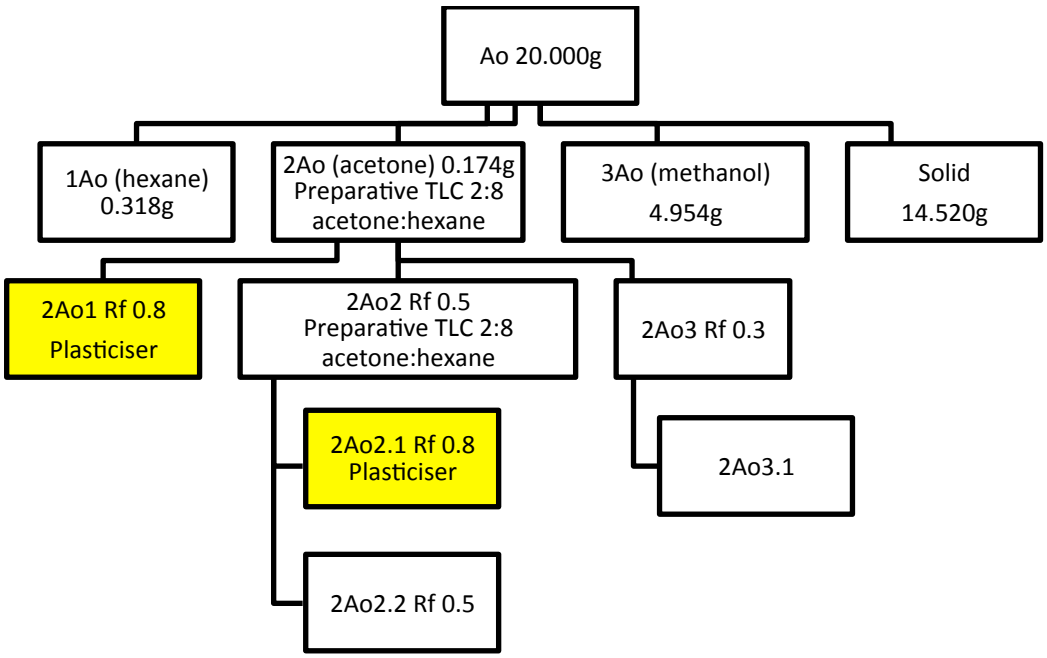


Figure 3-17 Summary of fractions from the first extraction of *Agelas oroides (Ao)*. Samples identified as plasticiser highlighted in yellow, Rf values relate to the method and solvent system used to separate a fraction or extract recorded in the previous box of the flow diagram.

*3.4.2.3 Re-extraction and isolation of antibacterial compounds from Agelas oroides (*Ao)*

Solvent extraction was performed for a sample of *Agelas oroides* that had been thoroughly cleared of visual foreign bodies as described in section 3.4.2.2. Once the extraction was complete with hexane, acetone and methanol, the extracts were compared to those from the first extraction (Figure 3-18). These samples were labelled as described in section 3.4.2.1 but labelled with an additional symbol (*) to differentiate from the previous extraction. The results were similar but not the same with differing concentrations of compounds visualised

in both also the yield was slightly worse at 19 % rather than 27 % for the first extraction. There are two possible explanations for this:

1. The sponge was ground to a finer powder as processing had been improved. Therefore more compounds are likely to be extracted due to the increased surface area of the crude material. Also as a larger amount of starting material was used, the ratio between the size of particle and the solvent would have been different.

2. Any potential contaminants were reduced by thoroughly removing visible debris with tweezers.

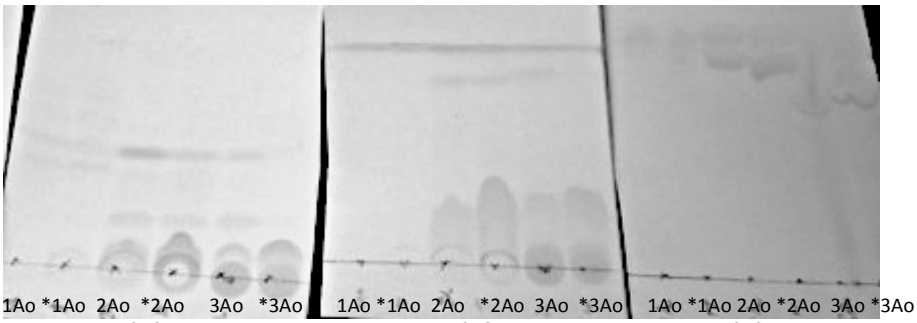


Figure 3-18 A digital image of TLC plates visualised at UV 254nm comparing the separation of *Agelas oroides* samples extracted using the three main solvents (hexane, acetone and methanol) from the first extraction (Ao) and extraction repeat (*Ao). Each plate shows left to right extracts 1Ao, *1Ao(hexane extracts), 2Ao, *2Ao (acetone extracts), 3Ao and *3Ao (methanol extracts) eluted in differing solvent systems with increasing polarity A-C. (A) = 3:7 (acetone : hexane). (B) = 1:9 (methanol : dichloromethane). (C) = 4:6 (methanol :dichloromethane).

Silica column chromatography was used for initial separation of material due to the large quantities of starting material. Extracts *1Ao, *2Ao and *3Ao were tested individually using analytical TLC to find the best conditions for column chromatography.

*3.4.2.4 Analysis of the chemical composition of the acetone extract of Agelas oroides (*2Ao)*

The acetone extract was separated using a silica column with a gradient of 2:8 - 5:5 - 1:0 (acetone : hexane). The fractions were grouped as Table 3-3 and combined by adding the contents of the similar fractions together and drying the final solution using the rotary evaporator. UV visualisation showed *2Ao3 (fractions 14-26) still contained a mixture of compounds (Figure 3-19), so it was separated further using preparative TLC in 2:8 (acetone : hexane) to produce another four fractions.

Table 3-3 Rf values and observed appearance of fractions of the acetone extract of *A. oroides* (*2Ao) visualised using analytical TLC 2:8 (acetone : hexane). The three grouped fractions were formed following separation of *2Ao using a silica column with a gradient of 2:8 - 5:5 - 1:0 (acetone : hexane).

Fraction name	Test tube number	Rf	Appearance
***2Ao1**	7-9	0.8	Colourless singular spot visible under UV(254nm)
***2Ao2**	12-13	0.5	Colourless mixed spot visible under UV(254nm)
***2Ao3**	14-15	0.5 + 0	Two coloured spots, visible under UV(254nm)
	16-18	0.4 + 0	Two coloured spots, visible under UV(254nm)
	19-23	0	Coloured spot on baseline, visible under UV(254nm)
	26	0.2 + 0	Two coloured spots, visible under UV(254nm)

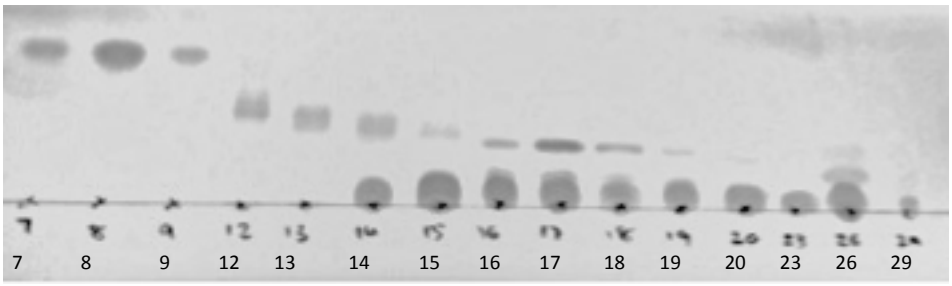


Figure 3-19 Digital image (UV 254 nm) of TLC separation (2:8 acetone : hexane) of fractions formed post the separation of the acetone extract of *A. oroides* using a silica column with a gradient of 2:8 - 5:5 - 1:0 (acetone : hexane). Samples labelled 14-26 chow clear mixtures of compounds.

*3.4.2.5 Analysis of the chemical composition of the hexane extract of Agelas oroides (*1Ao)*

The hexane extract was separated using a silica column with a gradient of 1:9 - 3:7 - 5:5 (acetone : hexane). *1Ao was grouped as the following five fractions (Table 3-4).

Table 3-4 Rf values, weight and observed appearance of fractions of the hexane extract of *A. oroides* (*1Ao), visualised using analytical TLC 1:19 (methanol : dichloromethane). The five grouped fractions were formed following separation of *1Ao using a silica column with a gradient of 1:9 - 3:7 - 5:5 (acetone : hexane).

Extract name	Test tube number	Weight (g)	Rf	Appearance
1Ao1	8-10	0.109	0.9	Yellow single spot visible under UV(254nm)
1Ao2	11-13	0.031	0.8	Orange singe spot
1Ao3	14-16	0.026	0.45	Colourless single spot visible under UV(254nm)
1Ao4	19-24	0.166	0.3	Yellow single spot visible under UV(254nm)
1Ao5	29-33	0.017	0.2	Faint yellow single spot visible under UV(254nm)

*3.4.2.6 Analysis of the chemical composition of the methanol extract of Agelas oroides (*3Ao)*

The methanol extract was separated using a silica column with a gradient of 8:2 - 5:5 - 0:1 (dichloromethane : methanol). The methanol extract proved a complex mixture and a variety of solvent systems were used for analytical TLC to best visualise different components.

The more polar composition of the solvent system of 8:2 (dichloromethane : methanol) (Table 3-6) meant *3Ao1 eluted higher up the plate than the less polar solvent system (2:8 acetone:hexane) (Table 3-5). This also shifted some of the previously stationary spots up into a visible area so that they could be differentiated. These were labelled *3Ao2, *3Ao3, *3Ao4. For further clarity, another slightly less polar TLC was completed to highlight any differences between fractions (Table 3-7). A comparative analytical TLC of *3Ao1, *1Ao and *2Ao in 2:8 (acetone : hexane) was also completed this showed that a fraction similar to that extracted in the less polar extracts was present in the methanol extract, thus indicating that the previous extractions were not complete.

Table 3-5 Rf values and observed appearance of fractions (combined to form *3Ao1) of the methanol extract of *A. oroides* (*3Ao), visualised using analytical TLC 2:8 (acetone : hexane). This fraction was formed following separation of *3Ao using a silica column with a gradient of 8:2 - 5:5 - 0:1 (dichloromethane : methanol).

Extract name	Test tube number	Rf	Appearance
***3Ao1**	16-20	0.4	Weak yellow band visible under UV(254nm)
	22-40	0	Variable colour spots trapped to the baseline

Table 3-6 Rf values and observed appearance of fractions of the methanol extract of *A. oroides* (*3Ao), visualised using analytical TLC 8:2 (dichloromethane : methanol). These four fractions were formed following separation of *3Ao using a silica column with a gradient of 8:2 - 5:5 - 0:1 (dichloromethane : methanol).

Extract name	Test tube number	Rf	Appearance
***3Ao1**	16-20	0.95	Weak yellow band visible under UV(254nm)
***3Ao2**	21-29	0.8-09	Orange spread band visible under UV(254nm)
***3Ao3**	30-33	0.7-09	Orange spread majority of colour lower than 3Ao2 visible under UV(254nm)
***3Ao4**	36-42	0.3-0.7	Smudged appearance, little colour visible under UV(254nm)

Table 3-7 Rf values and observed appearance of fractions of the methanol extract of *A. oroides* (*3Ao), visualised using analytical TLC 5:5 (acetone : dichloromethane). These three fractions were formed following separation of *3Ao using a silica column with a gradient of 8:2 - 5:5 - 0:1 (dichloromethane : methanol).

Extract name	Test tube number	Rf	Appearance
***3Ao2**	21-29	0.8+0.9	Two colourless bands highly visible under UV(254)
			Small faint orange smudge visible under UV(254nm)
	25-29	0.75	
***3Ao3**	33-36	0.5-0.7	Large orange smudge visible under UV(254nm)
***3Ao4**	37-46	0.3-06	Faint large orange smudge visible under UV(254nm)

Analysis of the purity of *3Ao2 by TLC showed two distinct bands (Table 3-7) therefore, it was further purified using preparatory TLC and separated into three further fractions. There was also some leftover solid that did not fully dissolve in the solvent system prior to elution through the column. This was retrieved, stored and labelled as *3Aosolid (Table 3-8).

Table 3-8 Yield of dried fractions of methanol extract of *A. oroides* (*3Ao).

Extract	Weight(g)
***3Ao1**	0.015g
***3Ao2.1**	0.022g
***3Ao2.2**	0.012g
***3Ao2.3**	0.078g
***3Ao3**	62.127g
***3Ao4**	oil
***3Aosolid**	0.062g

3.4.2.7 Assessing the antibacterial activity of Agelas oroides extracts against methicillin sensitive Staphylococcus aureus (MSSA)

Post fractionation selected samples were tested for activity against MSSA. The fractions chosen were those recovered in the highest quantities from the sponge, including one crude extract (*3Ao). Each sample was prepared and tested as described in (section 3.3.4). All samples shown in Figure 3-20 showed a statistically significant difference to the negative control of DMSO alone ($p < 0.05$ using Kruskal-Wallis). This demonstrated that *A. oroides* contained multiple different active fractions and therefore their constituent compounds were active against MSSA. Unfortunately, while this result is interesting, it did not provide quantifiable information, as the samples were not pure compounds. It did show that some of the extracts were almost as active as the positive control vancomycin and therefore some of the chemicals within the fractions must have potent activity.

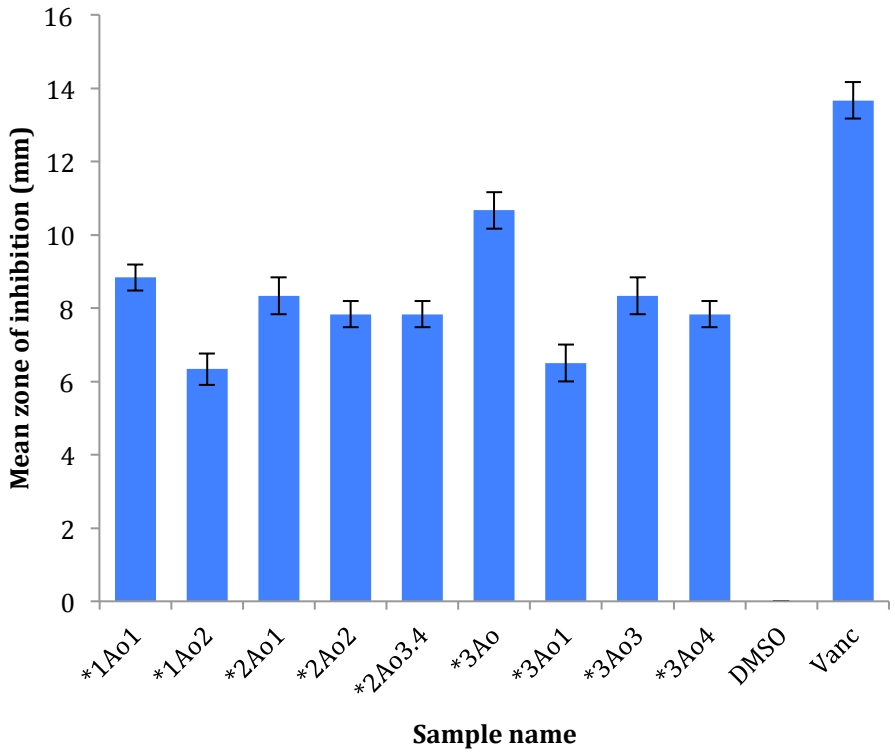


Figure 3-20 The area of inhibition of extract fractions (10 mg mL^{-1}) from *Agelas oroides* following well testing against MSSA. DMSO and vancomycin (Vanc) (10 mg mL^{-1}) were used as negative and positive standards respectively. (Error bars = Standard error, n = 3, mean zone of inhibition = average of two measurements for each zone).

The crude extract *3Ao showed the greatest inhibition of any of the samples tested at 11 mm, this was statistically higher than its constituent fractions tested ($p < 0.05$). This is noteworthy as it suggests that either the combination of active fractions within *3Ao worked in synergy to produce greater activity or something more active was not recovered it large enough quantities and therefore may not have been studied. It would not be appropriate to compare these results directly to literature, as the methods used are not standardised. *E.g.* a different strain of bacterium might have been used in different concentrations, which would therefore give different results. Although similar activity profiles against standards have been observed, for sponge extracts, using the disc diffusion method to measure zones of inhibition (Abdelmohsen *et al*. 2010; Rifai *et al*. 2005; Abbas *et al*. 2011) .

3.4.2.8 Purity testing of antibacterial active extracts against cholesterol and plasticiser standards.

The active extracts and initial crude samples of *A. oroides* extracts were compared to standards of cholesterol and plasticiser, found from the first extraction, no similarities were observed using analytical TLC. As the library of known compounds present in sponge samples was developed, through further extraction and separations, comparing samples to these standards prevented rediscovery of known compounds at an earlier stage of separation preventing work repetition.

3.4.2.9 Identification of extracts that displayed antibacterial activity using mass spectrometry as the principal method of spectroscopy

Although each fraction had been through some form of purification, it was observed that most fractions were not pure (Table3-9). Samples *2Ao1 and *3Ao1 looked very similar under TLC and appeared to be the purest of all fractions. These samples were analysed by MS at the School of Chemistry, Cardiff University.

Table 3-9 Summary of appearance and next processing step of active fractions and extracts isolated from *A. oroides.*

Fraction	Appearance under TLC	Next step
***1Ao1**	Single spot	MS
***1Ao2**	Streak	Separate further
***2Ao1**	Single spot	MS
***2Ao2**	Single spot	MS
***2Ao3.4**	Two spots	Separate further
***3Ao**	Crude	-
***3Ao1**	Single spot	MS
***3Ao3**	Multiple bands	Separate further
***3Ao4**	Multiple bands	Separate further

3.4.2.9.1 Fractions *2Ao1 and *3Ao1

These two fractions were analysed, via ES-MS and ^{1}H NMR, together as they appeared as single spots on TLC and had the same Rf under the same conditions. It was hypothesised at the time that they may represent the same compound.

ES-MS showed that they were not the same compound and that *2Ao1 was most likely a mixture of components. *2Ao1 showed two major peaks with the accurate masses of 304.1268 and 503.2129. It is possible these two peaks represent either the breakdown

product and its parent compound or a mixture of two compounds. The likely formulas produced gave no obvious clue of the identity of these compounds and the ^{1}H NMR analysis also provided little useable information apart from the fact aromatic protons were present. As the sample appeared a mixture, it could not be analysed through EI-MS, which meant it could not be compared to a database of compounds.

Fraction *3Ao1 appeared as a clear single peak after its first ES-MS with an accurate mass of 317.1068. None of the formulas produced seemed feasible and no matches occurred for any known compounds identified using *MarinLit*. The sample was analysed by EI-MS where different masses were observed compared to the ES-MS analysis (250.85 and 149.02); this may be due to chemical decomposition of the product. The EI-MS analysis indicated the presence of a bromopyrrole-alkaloid with a distinct isotope pattern. When matched against MassBank, it produced no likely structure, making this a potentially unknown active compound. Further tests would need to be completed to confirm a structure of the compound present but the sample was not recovered in a large enough quantity post separation and activity testing for further analysis. The information from this sample has been stored for future comparisons. Bromopyrrole-alkaloids are common products produced by marine sponges (Cafieri, Fattorusso and Taglialatela-Scafati 1998b; Eder *et al*. 1999) and have previously been found from extracts of *A. oroides* (Figure 3-2, Figure 3-3), therefore these results were expected.

3.4.2.9.2 Fraction *2Ao3.4.

Sample *2Ao3.4 was successfully separated into seven fractions. However, the weight of each fraction was insufficient to complete identification so the fractions were discarded.

3.4.2.9.3 Fraction *3Ao3.

Fraction *3Ao3 was further separated into five sub-fractions using preparative TLC 1:9 (methanol : dichloromethane). The yield obtained from this fractionation was notably poor (Table 3-10) at just 20% combined recovery from *3ao3. These sub-fractions were separated using a 1mm TLC plate that was potentially either overloaded with starting material or the extraction from the silica was not sufficient.

Table 3-10 Summary of yield of sub-fractions of *3Ao3.

Fraction	Weight(g)
***3Ao3.1**	0.010
***3Ao3.2**	0.018
***3Ao3.3**	0.031
***3Ao3.4**	0.119
***3Ao3.5**	0.016
Total	0.194

3.4.2.9.4 Mass spectrometry analysis of resultant active fractions

Mass spectrometry (MS) was completed on active extracts from Table 3-9. Samples 1Ao1, 1Ao2, 1Ao4 showed inconclusive results with no clear masses appearing in the lower range of less than *m/z* 500. All samples with clear masses are listed in Table 3-11.

Table 3-11 Mass spectrometry summary of active compounds isolated from *Agelas oroides.* Principal peaks highlighted in bold, with appropriate bromination marked in isotope pattern column.

Name	Mass identified (*m/z*)	Identification	Isotope patterns
***3Ao3.1**	413.1 and 803.5	Diisooctyl phthalate	
***3Ao3.2**	367.1	Unknown	
***3Ao3.3**	268.8, **339.9**, 341.9 and **409.9**	Unknown	Br1,Br2
***3Ao3.4**	268.8, 325.9 and **409.9**	Unknown	Br2
***3Ao4**	774.7	Unknown (and MW above 500)	

The only clear identification from the MS results was that of diisooctyl phthalate in *3Ao3.1, which is a common plasticiser contaminant. All low molecular weight unidentified extracts were searched in *MarinLit*, with the number of expected bromines from the isotope peak pattern, the expected mass (M + H) and the genus *Agelas*. This search provided no single matches for *m/z* 409.9 and *m/z* 339.9 providing 17 matches and 11 matches respectively. The peak identified at *m/z* 367.1 produced a single hit of agelanesin B. However, this is unlikely as the spectra (Figure 3-21) produced no brominated isotopic pattern. Unfortunately, due to lack of material further tests such as NMR could not be completed on these samples to confirm identification. It was also clear from the MS data that these samples were not pure due to the multiple mass peaks but *3Ao3.3 and *3Ao3.4 were still only recovered in 0.07% and 0.3% yield, respectively. While this is much higher than a concentration of 0.00001% that some active compounds have been isolated (Radjasa *et al*. 2007), the data sets are not entirely comparable due to the lack of purity of the fractions isolated. A complete extraction summary was produced to summarise the processes undertaken (Figure 3-22).

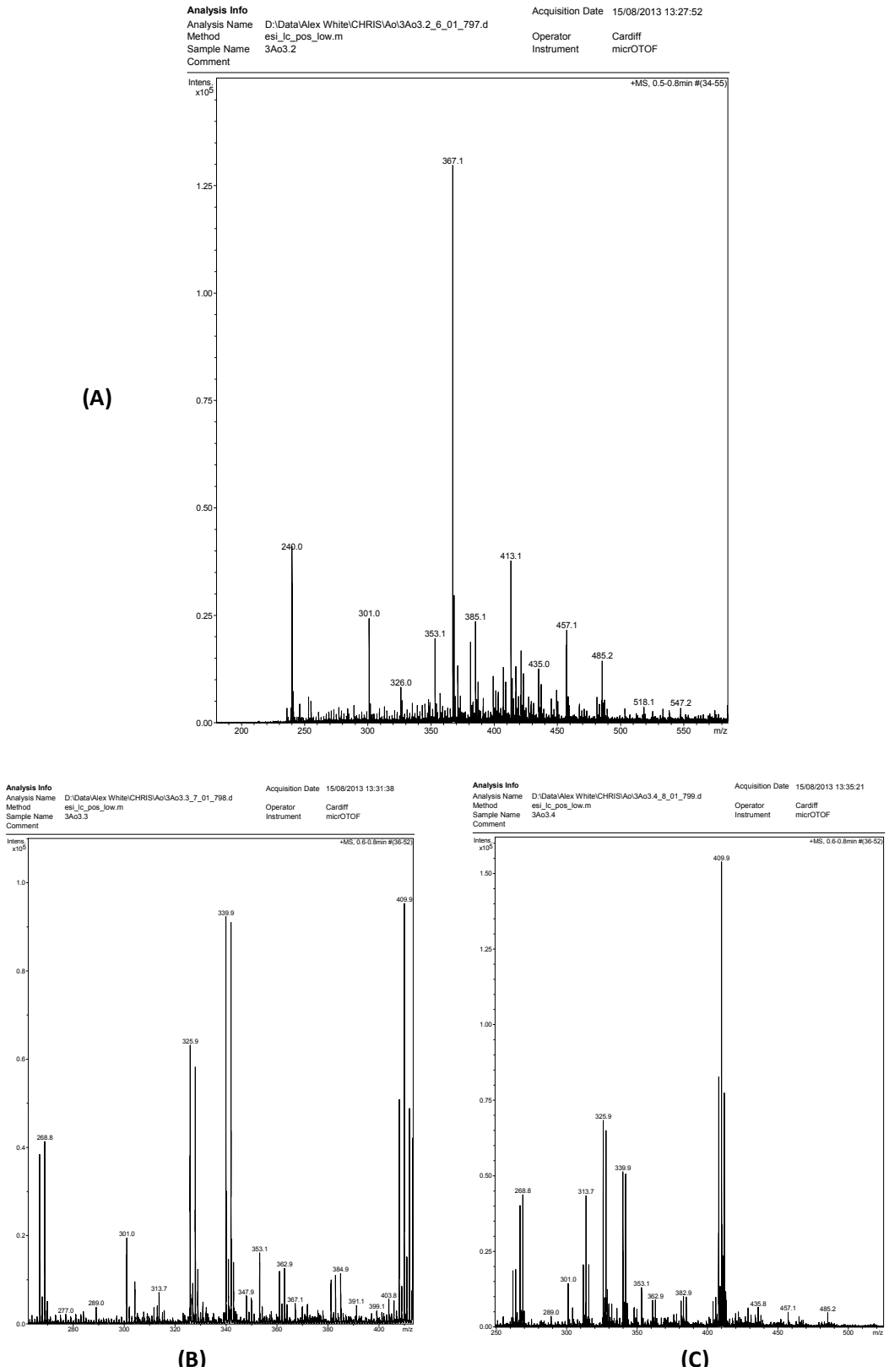


Figure 3-21 ES-MS spectra positive mode of: (A) 3Ao3.2, which displays no clear isotope pattern. (B) 3A03.3 showing single Br isotope pattern 339.9 and Br2 isotope pattern 409.9 and (C) 3Ao3.4 showing Br2 isotope pattern.

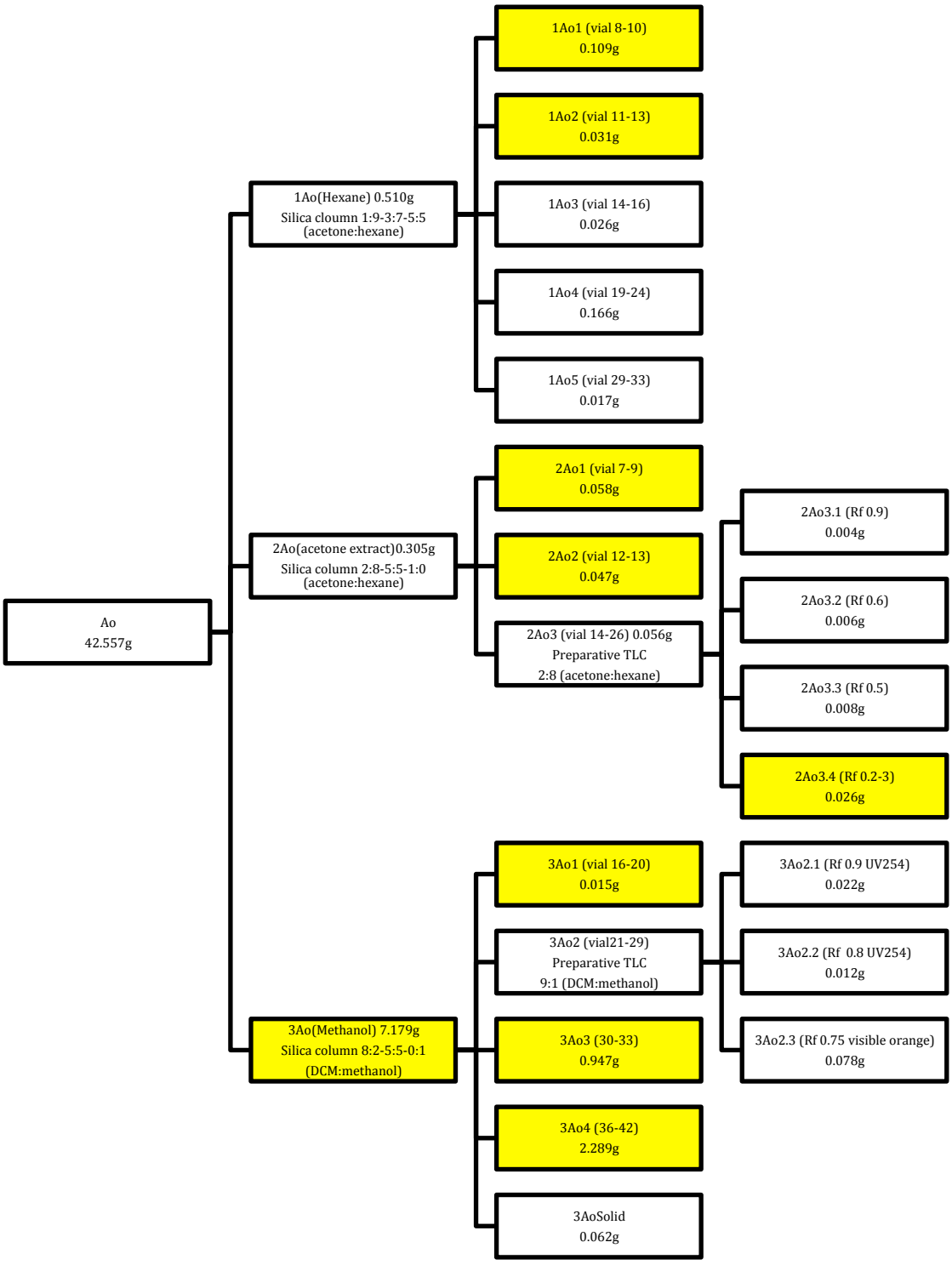


Figure 3-22 Extraction summary of *Agelas oroides* (*Ao). Samples highlighted in yellow were active against MSSA. Rf values relate to the method and solvent system used to separate a fraction or extract recorded in the previous box of the flow diagram. Final mass balance 3.921 g = 9% yield.

3.4.3 Isolation and identification of the chemical constituents of *Aplysina aerophoba (Aa)*

3.4.3.1 Solvent extraction of Aplysina aerophoba

Extraction of *A. aerophoba* followed the standard three-stage procedure (section 2.2.3) but also added a final stage, by completing a further extraction of the solid using Soxhlet extraction (section 3.3.3). This gave the crude extracts 1Aa, 2Aa, 3Aa and 4Aa, which were extracted with hexane, acetone, methanol and hot methanol, respectively (Table 3-12).

Table 3-12 Rf values, weight and observed appearance of extracts of *A. aerophoba* (Aa), visualised using analytical TLC 2:8 (acetone : hexane), under UV (254nm) and using iodine staining. Total Mass extracted 6.591g (41 % yield from dry sponge)

Extract	Weight(g)	Appearance	Rf	Simple TLC picture
1Aa	0.453	Yellow/orange oil	0.9 + 0.8	Two clear spots not visible under UV but highlighted by iodine staining.
2Aa	1.224		0.7 + 06	Two clear spots similar to those in 3Aa but lower concentration
3Aa	4.217	Orange/brown solid	0.7 + 0.6	Two clear spots, visible under UV. High concentration
4Aa	0.700		0.7+ 0.6+ streak	Two clear spots similar to those in 3Aa but lower concentration. Large streak. Complex mixture.

Due to the complexity of extracts, it was decided to only separate and attempt to characterise the mixtures 1Aa (hexane extract) and 3Aa (methanol extract).

3.4.3.2 Analysis of the chemical composition of the hexane extract of Aplysina aerophoba (1Aa)

Separation of the two principal spots, highlighted in (Table 3-12) was completed using preparative TLC with 2:8 (acetone : hexane) which provided sufficient separation of the two principle spots to yield fractions 1Aa1 (0.029 g) and 1Aa2 (0.093 g). Fraction 1Aa1 appeared pure by analytical TLC, therefore it was sent for structural characterisation. However, 1Aa2 appeared a complex mix and was separated further.

3.4.3.2.1 Fraction 1Aa1

Fraction 1Aa1 was prepared for ^{1}H NMR in DMSO (10 mg mL^{-1}), which gave a spectrum typical of a sterol such as cholesterol (Figure 3-23, Table 3-13). This was confirmed, using

stock cholesterol (Sigma-Aldrich Ltd, UK) as a standard, via ^{1}H NMR and TLC combined with low resolution MS. Sponges are known sources of a variety of sterols and cholesterol is known to be present in a large number of species (Santalova *et al.* 2004).

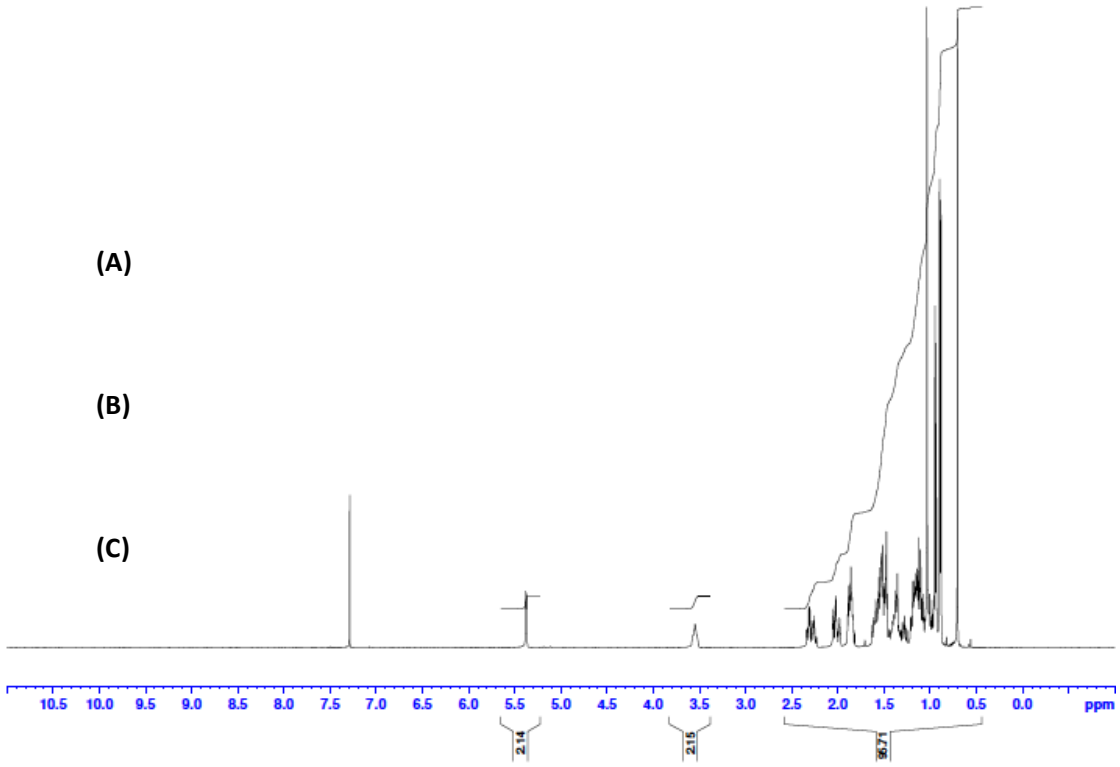


Figure 3-23 (A) ^{1}H NMR taken from Schfinder; (B), ^{1}H NMR of extracted Cholesterol. (B) ^{1}H NMR of authentic cholesterol and structure of cholesterol with matching predicted peaks.

Table 3-13 Summary of the matching chemical shifts of stock cholesterol and extracted compound considered to be cholesterol.

Atom number	Shift (ppm)	Type	H's
11	5.37	dd	1
16	3.58	m	2
17	2.23	m	4
9, 10	2.04	m	4
3, 14, 15	1.82	m	7
6, 8, 10, 23	1.54	m	19
2, 3, 14, 20, 21	1.44	m	9
4, 9, 21, 22	1.38	m	15
25	1.25	s	7
5, 7, 20	0.96	m	4
27	0.91	d	6
24	0.86	d	6
28	0.86	d	6
18	0.68	s	6

3.4.3.2.2 Fraction 1Aa2

Fraction 1Aa2 appeared a complex mixture, via TLC, and was further separated using preparative TLC 1:9 (acetone : hexane), providing three further fractions. Fractions 1Aa2.1 and 1A2.2 appeared as single spots, when analysed by TLC (Table 3-14) and the investigation was continued to enable characterisation. Fraction 1Aa2.3 was discarded as it appeared a complex mixture and insufficient quantity was isolated.

Table 3-14 Rf values, weight and observed appearance of sub-fractions from the hexane extract of *A. aerophoba* (1Aa2) when visualised using analytical TLC in 1:9 (acetone : hexane). Fractions formed post separation using preparative TLC 1:9 (acetone : hexane).

Fraction	Weight (g)	Rf	Appearance
1Aa2.1	0.023	0.4	Colourless only visible with iodine staining
1Aa2.2	0.031	0.3	Colourless only visible under UV
1Aa2.3	0.011	0	Colourless baseline spot. Looked a mix on further TLCs.

3.4.3.2.3 Fraction 1Aa2.1

Analysis by ^{1}H NMR of 1Aa2.1 produced a complex spectrum, which suggested the compound contained no aromatic protons (hence no visibility under UV), multiple carbon-carbon double bonds and carbon-oxygen or nitrogen single bonds. The highest intensity MS

peak was found at 396.36 and did not contain the typical bromine isotope peak found in brominated alkaloids. The accurate mass obtained was 396.3616 and 396.3603 rendering the most likely empirical formula of $C_{25}H_{48}O_3$. A search performed on the database *SchiFinder* identified clionasterol (Figure 3-24) as the most likely candidate. Clionasterol acetate is a sterol that is commonly synthesized by a multitude of marine sponge species (Sawangwong *et al*. 2008), it is named after the sponge from which it was first discovered *Cliona. sp* (Valentine and Bergmann 1941).

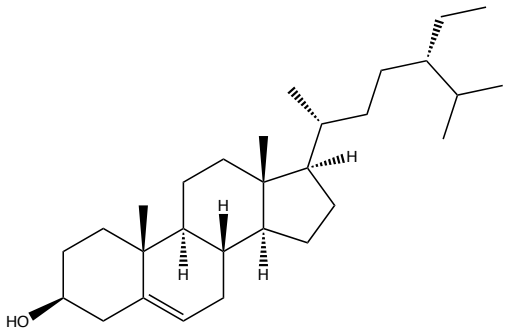


Figure 3-24 Structure of clionasterol

3.4.3.2.4 Fraction 1A2.2

The ^{1}H NMR of 1Aa2.2 provided a clear spectrum of a phthalate plasticiser consistent with the spectrum, found in A. oroides (Table 3-10), which was indicative of a plasticiser. This sample was kept for use as a standard in further experiments.

3.4.3.3 Assessing the chemical composition of the methanol extract of Aplysina aerophoba (3Aa)

Analytical TLC was completed with the intent of isolating the two principal spots of the methanol extract of *A. aerophoba*, highlighted in (Table 3-12). Close scrutiny of the TLC showed four distinct fractions, which were separated using preparative TLC with 2:8 (acetone : hexane) (Table 3-15).

Table 3-15 Rf values, weight and observed appearance of fractions from the methanol extract of *A. aerophoba* (3Aa) visualised using analytical TLC in 2:8 (acetone : hexane). Fractions formed post separation using preparative TLC 2:8 (acetone : hexane).

Fraction	Weight (mg)	Rf	Appearance
3Aa1	18mg	0	
3Aa2	7mg	0.3	
3Aa3	37.1mg	0.5	Two bands visible under UV merged together in the middle
3Aa4	17mg	0.9	

Separation of 3Aa3 proved impossible with preparative TLC, thus larger quantities of sample were eluted using column chromatography for improved yield and better separation. A sephadex column, 10:10:80 (hexane : methanol : acetone), produced 96 fractions of which fractions 25-36 indicated the presence of the compounds targeted from 3Aa3 but did not separate them. However, combining these fractions gave a larger quantity of material (511mg).

Table 3-16 Rf values, and observed appearance of sub-fractions from a methanol fraction of *A. aerophoba* (3Aa3) when visualised using analytical TLC 1:9 (methanol : dichloromethane). Fraction formed post separation using a Sephadex column 10:10:80 (hexane : methanol : acetone).

Extract	Test tube number	Rf	Appearance
	20	0.5	Clear spot
	23	0.3 +0.55 +0.6	Faint spots and clear spot at 06
3Aa3	25-36	0.5 + 0.6	Two clear spots visible under UV

3Aa3 was further separated by preparative TLC 1:9 (methanol : dichloromethane) (Table 3-17). The products of the preparative TLC plate were combined, producing three new fractions labelled as 3Aa3X, 3Aa3Y and 3Aa3Z. TLC against standards from the earlier separation identified that two different fractions both contained the standards but also some impurities so they were combined to form 3Aa3A (Table 3-17) Due to the small quantity of material obtained from a separate fraction (3Aa3Z), it was immediately analysed using spectroscopic techniques.

Table 3-17 Rf values, and observed appearance of sub-fractions from a methanol fraction of *A. aerophoba* (3Aa3) when visualised using analytical TLC 1:9 (methanol : dichloromethane). Fraction formed post separation with preparative TLC 1:9 (methanol : dichloromethane).

Extract	Weight(mg)	Rf	Appearance
3Aa3X	130mg (combined with 3Aa3Y to form 3Aa3A)	0.2	Faint band visible under UV. Appeared as complex mixture containing both 3Aa3.1 and 3Aa3.2)
3Aa3Y	130mg (combined with 3Aa3X to form 3Aa3A)	0.3	Clear band visible under UV. Appeared as complex mixture containing both 3Aa3.1 and 3Aa3.2)
3Aa3Z	8mg	0.9	Clear band visible under UV

3.4.3.3.1 Fraction 3Aa3Z

Analysis by ^{1}H NMR proved inconclusive but showed the likelihood of two hydrogen atoms attached to an aromatic ring and a methyl group attached to a CH_2 group. MS analysis showed a baseline peak at *m/z* 230.14 and a lower intensity peak at the higher value *m/z* 244.18. This indicated the possible presence of impurities in the sample and that it most likely consisted of more than one compound. An intense MS peak observed at *m/z* 91 suggested a tropylium cation (Figure 3-25) and hence the presence of an aromatic ring. (Rylander *et al*. 1957).

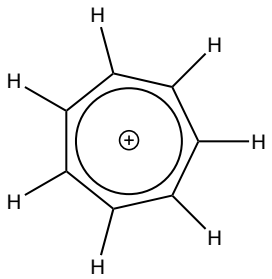


Figure 3-25 Structure of tropylium cation (Rylander *et al*. 1957)

Unfortunately, this sample could not be fractionated further due to its low yield. Also, this result could not be compared to MassBank as fragmentation and library comparison can only be completed for pure compounds and no hits were found when limited by the genus *Aplysina*.

3.4.3.3.2 Fraction 3Aa3A

Further separation of 3Aa3A was completed, using preparative TLC 3:7 (methanol : dichloromethane), which highlighted five separate bands (Table 3-18).

Table 3-18 Rf values, weight and observed appearance of sub-fractions from a methanol fraction of *A. aerophoba* (3Aa3A) when visualised using analytical TLC 1:9 (methanol : dichloromethane). Fraction formed post separation with preparative TLC 3:7 (methanol : dichloromethane).

Extract	Weight (g)	Rf	Appearance
3Aa3.1	0.008	0.1	Faint single band visible under UV
3Aa3.1.5	0.003	0.2	Clear single band visible under UV
3Aa3.2	0.008	0.25	Clear single band visible under UV
3Aa3.3	0.005	0.3	Clear single band visible under UV
3Aa3.4	0.007	0.5	Faint single band visible under UV

When eluted against TLC standards, 3Aa3.1 and 3Aa3.2 appeared the same as those originally identified (Table 3-12). These two samples along with 3Aa3.4 were chosen for further identification, 3Aa3.1.5 and 3Aa3.3 were discarded due to poor yield.

Analysis by ^{1}H NMR of fractions 3Aa3.1 and 3Aa3.2 produced very similar spectra, which suggested neither contained aromatic rings but may contain carbon-carbon double bonds. Mass spectra analysis produced similar results for the two compounds 3Aa3.1 and 3Aa3.2 with both showing almost identical principal peaks at *m/z* 333.85 and *m/z* 333.86 implying they may indeed be the same compounds. Electrospray MS indicated both compounds were indeed mixtures and they could not be separated further due to low yield. The masses obtained did not match any brominated alkaloids previously extracted from *A. aerophoba* and no hits appeared when limited by the genus *Aplysina* in *MarinLit*.

^{1}H NMR of 3Aa3.4 was also completed. Again, this spectrum indicated that of plasticiser, highlighted by the characteristic multiplet in the aromatic region. The spectra was however different to that of 1Aa2.2 showing a different composition in the area of branching alkyl groups. Further determination of this plasticiser proved impossible due to the relatively low yield and generally higher quantity of product needed for ^{13}C NMR. The discovery of this plasticiser seemed particularly unusual as dialkyl phthalate esters are hydrophobic compounds and therefore expected to be extracted in the hexane fraction. The presence in the methanol extract may be for one of two reasons:

1. Contamination during extraction and isolation or the pipette bulb.
2. Incomplete extraction during the hexane extraction and saturation of the solvent.

A complete extraction summary of *Aplysina aerophoba* was produced to summarise the processes undertaken (Figure 3-26).

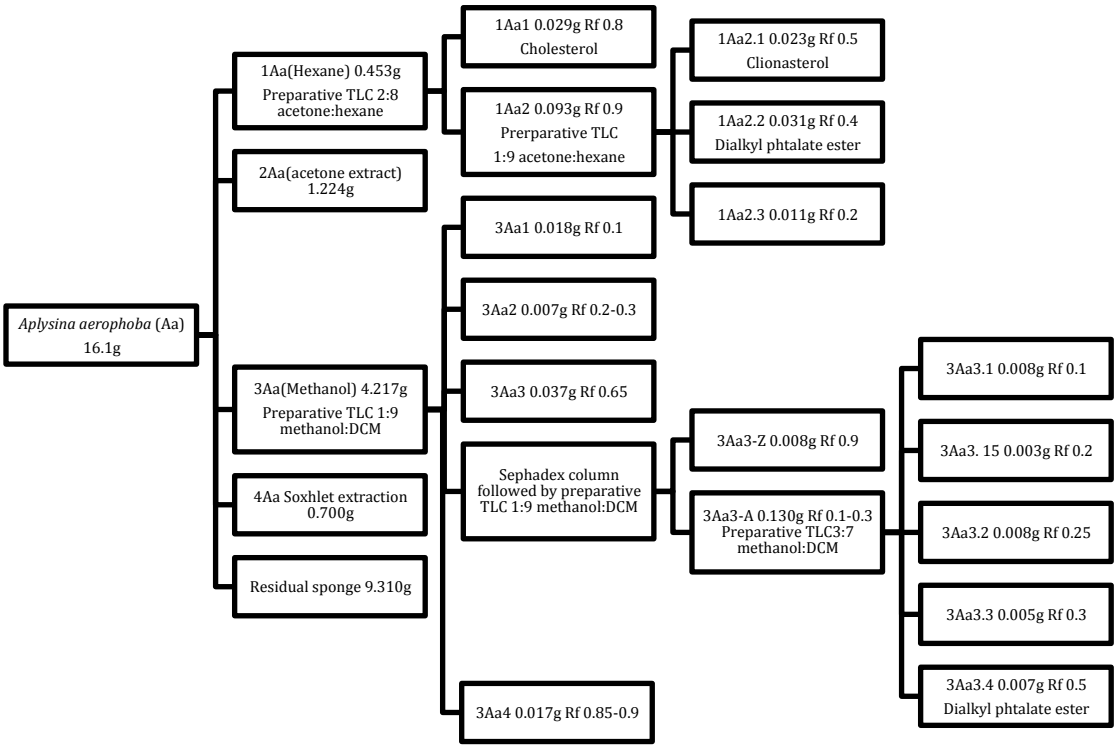


Figure 3-26 Extraction summary *Aplysina aerophoba* (Aa), including weights of products (extracts and fractions), methods and solvents systems used for separation.

3.4.4 Discussion of preliminary separation methods

While the methods employed proved successful at identifying compounds, they were not activity targeted and were hindered by rediscovery. This was expected and was circumvented in future experiments by employing an activity-targeted method of pre-screening preventing time and resources wasted on rediscovery (Hughes and Fenical 2010).

Utilising the knowledge gained from these preliminary experiments an ideal pre-screen for a drug lead compound would offer the following:

- The activity of each extract/fraction before further separation.
- The molecular mass: ensuring the compound is drug like with MW of less than 500 and for comparison to databases to prevent rediscovery.
- MS fragmentation pattern for more robust information on the compound.
- Effective for each extract for all sponges from non-polar to polar.
- Use as little material as possible.

Once the pre-screen was complete, the molecule itself could be targeted for removal, using methods that recover a higher yield such as Counter Current Chromatography (CCC), which theoretically provides virtually no loss of material due to its lack of solid phase (Sutherland and Fisher 2009).

In subsequent studies, it was decided that screening for activity from each extract from each sponge collected should be conducted all at once to allow direct comparisons between species and their chemical composition. The most commonly used method for separation of natural products is HPLC (Debitus *et al*. 1998; Latif and Sarker 2012; Bucar *et al*. 2013), although the solvent systems can be difficult to perfect, to achieve good separation, and are not always reproducible when scaled up (Sutherland and Fisher 2009). Also any non-UV active samples such as cholesterol and clionasterol, identified in these preliminary experiments would be missed via the UV-detector (Houssen and Jaspars 2012) unless other methods of detection were used. It is also possible that hexane extracts would not dissolve for testing or irreversibly bind to the solid phase, which would contradict the complete chemical screen concept of this project. Another problem with the hexane extract is that even if separation proved possible for each fraction via HPLC, activity testing would have to completed using the well method or the disc diffusion method of activity testing. Neither of these tests works for non-polar compounds, as they cannot diffuse through the agar (Hamburger and Cordell 1987).

One traditional method that avoids the dissolution and diffusion problem associated with HPLC is the use of direct bioautographic techniques (Hamburger and Cordell 1987). This would allow the direct visualisation of activity on a TLC plate, identifying where activity has eluted along the TLC plate and the compounds responsible. Mass spectrometry analysis

directly from a TLC plate is now possible and by coupling this to bioautgraphic data, rapid identification of antibacterial compounds from natural products is possible (Kasote *et al*. 2015).

3.5 Conclusion

The results from this chapter demonstrated that marine sponges are chemically complex organisms by the extraction and identification of multiple different classes of compound. This was predictable with sponges known to be prolific producers of active secondary metabolites (Valentine and Bergmann 1941; Lira *et al*. 2011). The techniques used to test and extract active compounds from a sponge were effective with multiple known and expected (Sawangwong *et al*. 2008) compounds being identified. While these techniques have proved useful at identifying known compounds, yield limitations have hindered the identification of any novel compounds. This was expected with the separation techniques employed thus far as many compounds may irreversibly bind to the solid stationary phase used for separation (Sutherland and Fisher 2009). These initial method development experiments have highlighted that it is much easier to identify known compounds than unknowns and this realisation was used to the advantage of this project with the introduction of pre-screens as a method of preventing rediscovery as early as possible.

The techniques employed in these first extractions were also blind with activity appearing through luck, post isolation of compounds, rather than conducting bio-assay guided fractionation. It was notable for the methanol extract of *Agelas oroides* that some activity was potentially lost through separation (section 3.4.2.9). All future testing was proceeded by an activity-guided pre-screen, which used as little material as possible, to gather useful information.

The actual extraction methods proved successful with yields of 27 % (Ao), 19 % (*Ao) and 41 % (Aa) respectively, with Soxhlet extraction adding an extra 3.5% to the yield of the *Aplysina aerophoba* extraction. The yield achieved appears to vary between species with almost double the material extracted from *Aplysina aerophoba* as apposed to *Agelas oroides* even with Soxhlet extraction discounted. This will be compared further in future experiments although the yields appeared promising with only 4% (Youssef *et al*. 2013), 3% and 7% (Kochanowska *et al*. 2008) and 8% (Carney *et al*. 1993) achieved in a similar studies. Soxhlet extraction will not be employed for future samples as it provided materials that were too polar to be dissolved for accurate activity testing. It was also noted that compounds from Soxhlet extraction did not separate well on TLC, possibly due to their polarity or because they were decomposed or chemically altered by the heat involved in the process (Bucar *et al*.

2013). The relative yield (3.5 %) was also comparatively low and seemed to contain some similar compounds that were previously extracted. It was decided future experiments would only utilise Soxhlet extraction when significant antibacterial activity was found in a methanol extract and not enough material was available.

Chapter 4
Antibacterial activity of unstudied Welsh and Greek sponges

4 Antibacterial activity of unstudied Welsh and Greek sponges

4.1 Chapter introduction

4.1.1 Welsh sponges

The Welsh coastline provides a unique opportunity to exploit unstudied marine sponges. The majority of research has been completed in the naturally rich tropical climates. Whilst some temperate sponges have been chemically characterised, research on British sponges has been minimal. A search was completed on Thomson Reuters Web of Science in 2012 comparing the number of matches for 'Marine Sponge' and 'Tropical or Temperate'. In total, 414 matches were found for tropical marine sponges and 164 for temperate marine sponges. These results determined that over double the amount of published research on marine sponges has concentrated on tropical species rather than temperate sponges. This same search was completed again in 2015 producing a similar pattern with 463 matches for tropical sponges and 188 matches for temperate sponges.

An ocean is considered temperate if its temperature ranges between 4 - 24 °C (Peel *et al*. 2007). This covers a vast sea area and unusually groups the quite dissimilar Mediterranean Sea with the North Sea (Figure 4-1). Most previous temperate research has been completed in the Mediterranean (Fattorusso and Taglialatela-Scafati 2000; Thoms *et al*. 2004; Muscholl-Silberhorn *et al*. 2008; Ferretti *et al*. 2009; Casapullo *et al*. 1993) and other seas at the warmer end of the scale, therefore concentrating on a colder temperate sea, like that surrounding Wales, narrows this field down further.

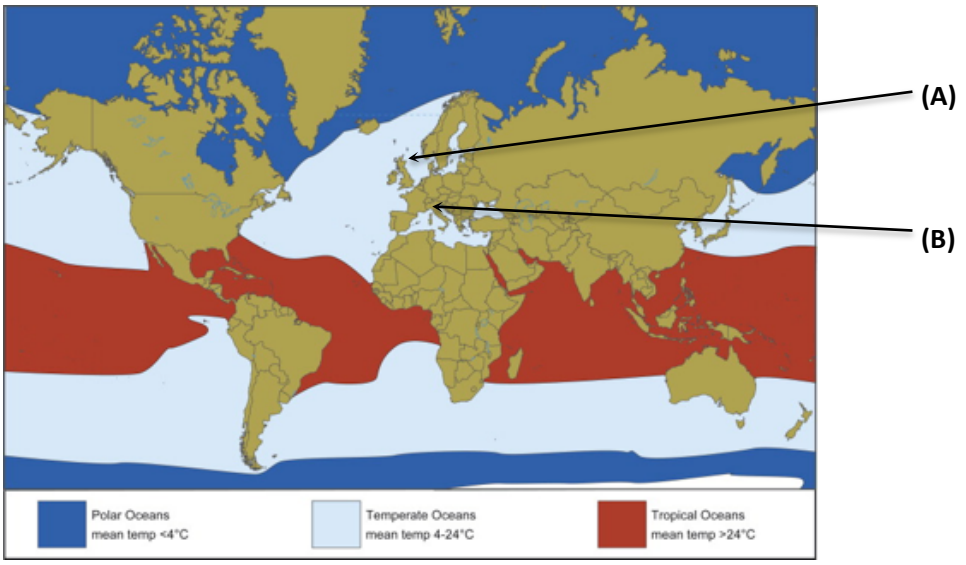


Figure 4-1 Ocean temperatures adapted from Peel *et al* (2007). (A) North Sea. (B) Mediterranean Sea.

Although temperate waters may not be as naturally rich in diversity as tropical waters (Figure 4-2), they still show well above average variation in species. This is the case for both Wales and Greece.

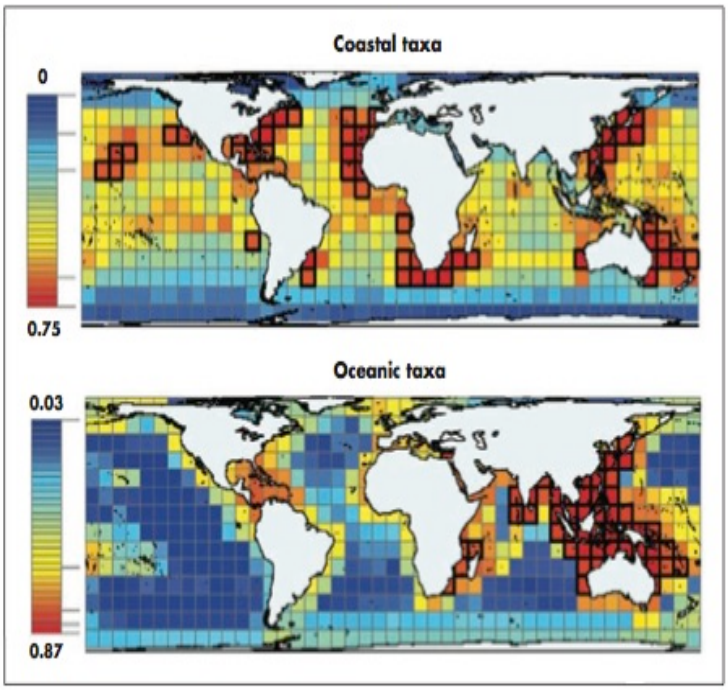


Figure 4-2 Records of 11,000 marine species from the *Ocean Biogeographic Information System of the Census* revealing hot spots of species diversity. Red colouration indicates areas of high diversity. Horizontal tick marks to the right of the colour ramps indicating quartiles of diversity show it takes more diversity in coastal regions to qualify for a high ranking taken from Ausubel *et al*. (2010)

4.1.1.1 Recent research on UK marine sponges

The first major survey of UK marine sponges since the 19th century was undertaken between 2008 and 2011 by Goodwin and Picton (Goodwin *et al*. 2011; Goodwin and Picton 2011) which summarised the variety of species, which were prevalent in UK waters. This research group was contacted and they produced a summary of sponges in the UK. This summary was developed into a collection list by limiting by the following criteria:

- Availability of sponge: whether the sponge was thought to be prevalent, with input by the research team from Skomer and C. Goodwin (Department of Natural Sciences, National Museums Northern Ireland) and to determine whether each species was collectable (average size, type of sponge etc.).
- Characterisation of sponge: whether the sponge had been previously studied and chemically characterised.

Once narrowed down, a final collectable list was produced ready for sampling.

4.1.2 Bioautographic chromatography

Bioautographic chromatography is an analytical technique that can identify growth inhibitory effects of compounds, separated by thin layer chromatography (TLC), upon microorganisms. Bioautographic techniques can be divided into direct and indirect methods. Direct bioautography involves the direct application of a bacterial suspension onto a TLC plate, giving quick and simple indication of activity. An indirect method involves layering agar on top of a TLC plate and the compounds having to pass through the agar to display activity. A key advantage of the direct bioautographic method is that it has superior sensitivity to the indirect method, allowing detection of antibacterial activity in lower concentrations (Begue and Kline 1972). This was particularly important in this study, as the amount of starting material was limited and was vitally important in subsequent identification steps.

Bioautography is a traditional technique that is not commonly employed in modern natural product activity testing but has the advantage over other more instrumental methods such as HPLC followed by activity testing, due to its simplicity in not having to manually test every separated fraction. Bioautography 'highlights' every active antibacterial compound unlike HPLC, which may miss any non-UV active antibacterial compounds (Houssen and Jaspars 2012). It is also effective for non-polar compounds, due to a direct effect on bacteria, unlike

agar diffusion methods where non-polar compounds cannot penetrate the agar due to their polarity (Kasote *et al*. 2015).

The technique employed by this project used a tetrazolium salt, Iodonitrotetrazolium chloride (INT) (Sigma Aldrich Ltd, UK), which is a good substrate for enzymatic reactions of actively metabolising bacteria (Begue and Kline 1972). INT turns pink upon detection of dehydrogenase activity, which indicates actively growing, and hence metabolising, bacteria (Begue and Kline 1972). Once layered onto a TLC plate, any areas stained pink had live bacteria growth and any area with a clear zone had no bacteria growth therefore showing the separated compound displayed antibacterial activity (Begue and Kline 1972). The overlay bioautographic technique was chosen in this study as it allowed easy localization of activity and an effective visual comparison of growth inhibition activity differences between the sponge samples. Bioautography has been employed previously to explore the activity of MNP's from alga (Caccamese *et al*. 1989) and other natural products (Valgas *et al*. 2007).

The antibacterial assay used in this project was developed to determine if any of the separated sponge extracts possessed activity against MSSA (NCIMB 9518) or *E. coli* (NCIMB 12210), covering both major classifications of cell envelope, Gram-positive and Gram-negative bacteria, respectively (section 1.5.1). Both Gram classifications were chosen, as activity against one does not indicate activity against the other (section 1.5.1). Other research projects have found that less antibacterial activity is often observed against Gram-negative bacteria when testing natural products (Abdelmohsen *et al*. 2010; Abbas *et al*. 2011), due to the increased complexity of their cell wall (section 1.5.1).

MSSA (NCIMB 9518) is a methicillin sensitive strain of *Staphylococcus aureus* and was chosen for preliminary activity testing instead of its methicillin resistant counterpart (MRSA), as this strain may be resistant to one or more compounds isolated from sponge. Activity testing against *E. coli* (NCIMB 12210) is also important; as it is a medically relevant strain with infection on the rise in healthcare environments (Davies *et al*. 2013). Both strains chosen are clinically relevant and have been used to assess the antimicrobial activity of UK sponge related microbes (Flemer *et al*. 2012).

4.1.3 Quantifiable activity assays

The direct bioautographic technique is a useful method for visualising growth inhibition activity in a sample, but it is not quantifiable. Other techniques were implemented to

quantify the activity of an extract. The two most commonly used laboratory techniques for studying antibacterial activity are the disc diffusion method (Ericsson *et al*. 1960) and the microdilution method (Hughes and Fenical 2010; Ericsson and Sherries 1971).

4.1.3.1 Disc diffusion assay

The disc diffusion assay involves impregnating a filter disc with a test substance. This is then placed on a lawn of test bacteria and incubated. Antibacterial activity is measured by the diameter of growth inhibition on the agar plate with zone of inhibition correlating directly to antibacterial activity (Bauer *et al*. 1966; Ericsson *et al*. 1960). A limitation of this method is some substances may have different diffusion coefficients across the agar, due to their differing polarity (Ericsson *et al*. 1960; Schwalbe *et al*. 2007). Consequently comparisons between sequential solvent extracts cannot be reliably performed as they have been purposely separated via their polarity. Furthermore, hexane extracts may not be suitable for analysis using this method due to their non-polar nature, as they may not diffuse through the agar for visualisation of activity.

4.1.3.2 Microdilution assay

In the microdilution assay, extracts are serially diluted along a microtitre plate and incubated with the test bacterium inoculated into each well (Ericsson and Sherries 1971). The minimum inhibitory concentration (MIC) is calculated by measuring a change in the optical density within a well or using a dye to visualise bacterial growth (Schwalbe *et al*. 2007). The disadvantage of this method is that some of the sponge extracts were highly coloured and any changes in optical density were not always clear. This project resolved this with the use of INT to dye actively metabolising bacteria. It was also observed that some samples, notably the hexane extracts, would not dissolve evenly prior to dilution, and therefore could not be reliably tested. Using this method, the minimum bactericidal concentration (MBC) was also calculated to give the concentration of extract needed to kill a chosen bacterium. All extracts in this study, that solubilised in DMSO or water, had their MIC and MBC calculated using the microdilution assay.

MRSA (NCTC 11939), originally isolated from a Hospital environment, was selected for the example of gram-positive bacteria for the microdilution assay as activity had already been observed against MSSA (NCIMB 11939) using the bioautgraphic technique. Completion of a

quantifiable technique against a clinically relevant resistant strain of bacteria provided data with greater impact.

4.2 Chapter aim and objectives

The primary aim of this chapter was to extract and identify active fractions from unstudied marine sponges. The objectives of this chapter were to:

- Describe the collection of a variety of Greek and Welsh sponge samples. This includes unstudied species and those found in differing conditions such as depth, location and competitive environment.
- Perform sequential extraction on all Welsh and Greek samples providing preliminary separation of sponge material (section 2.2.3).
- Investigate antibacterial activity against MSSA and *E. coli* using bioautographic methods and pinpoint compounds of interest for isolation and identification.
- Determine quantifiable MIC tests on each active extract to verify results of bioautographic assay.

4.3 Methods

4.3.1 Sample extraction

4.3.1.1 Greek sample extraction

For each sample, 0.5 g of material was extracted in 50 mL of solvent. The sequential method of extraction was applied (section 2.2.2), using solvents of increasing polarity to obtain three different extracts for each sponge sample. The samples were shaken overnight rather than stirred. The containers were placed horizontally to achieve optimum agitation.

Each extraction was aided by a sonic bath, for 20 min at 20 °C (FB15047 sonicating bath, Fisherbrand, UK),

4.3.1.2 Welsh sample extraction

All material collected for each Welsh sample was used for extraction, except for the material stored for bacterial cultivation (section 2.2.1.2). Extraction was completed for the Welsh samples using the standard solvent extraction method (section 2.2.2), the samples were also placed in a sonic bath, for 20 min at 20 °C (FB15047 sonicating bath, Fisherbrand, UK), to aid extraction by agitating the mixture.

4.3.2 Bioautographic technique

The test extracts and were dissolved to 20 mg mL^{-1}, in their extraction solvent. An aliquot of 10 µL was pipetted onto an analytical TLC plate (Merck KGaA, Darmsradt, Germany) and allowed to dry. The solvent systems used for elution were based upon the solvent used for extraction. The solvent systems used were 8:2 (hexane : acetone) for hexane extracts, 6:4 (hexane : acetone) for acetone extracts and 8:2 (dichloromethane : methanol) for methanol extracts, unless otherwise stated. Each plate was then eluted at room temperature in a glass chamber with 50 mL of solvent system decanted into the bottom. Upon elution, a digital photograph was taken of the plate in visible light and under UV at two wavelengths (254 and 365 nm) (UVGL-58 handheld lamp, UVP, Canada).

All extracts were tested for activity against MSSA (NCIMB 9518) and *E. coli* (NCIMB 12210). Bacterial samples were prepared as described in section 2.5.3 with all manipulations

performed in a category 2 laminar flow cabinet. Bacterial suspensions were serially diluted (1:10), to form suspensions of 10^6 CFU mL^{-1}, in fresh sterile nutrient broth.

The diluted bacterial suspension was spread evenly over an eluted TLC plate using a sterile roller, before being allowed to dry in the laminar flow cabinet. Dry plates were placed in a hermetically sealed, humid, polyethylene container before being incubated at 37 °C for 16 h. TLC plates were evenly sprayed with a 2 mg mL^{-1} solution of INT, before drying in a laminar flow cabinet for 10 min. Dry plates were placed back in the polyethylene container and incubated at 37 °C for a further 4 h. Clear zones on the plates indicated inhibition of bacterial growth. The Rf of any zone of activity was recorded and compared to the digital photograph taken upon elution of the TLC (Figure 4-3).

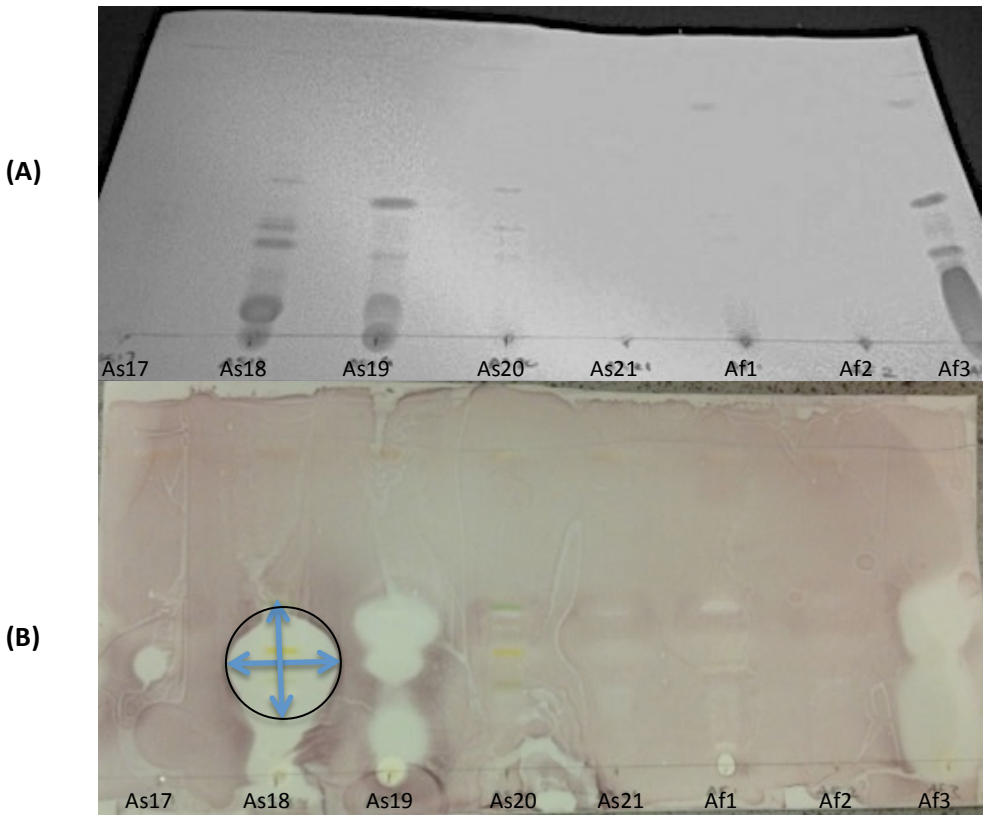


Figure 4-3 (A) An example of TLC plate eluted with 2:8 (acetone : hexane) showing separated components of extracts (20 mg mL^{-1}) visualised by UV light (256nm). (B) The same TLC plate following application of the bioautographic technique with clear zones of inhibition over individual spots of separated extracts. Label and species = As17, As21 and Af2 (*Chondrosia reniformis*), As18 (*Aplysina aerophoba*), As19 and Af3 (*Agelas oroides*), As20 (*Chondrilla nucula*), Af1 (*Spirastrella cunctatrix*).

4.3.3 Disc diffusion method

Müller Hinton agar was prepared according to the manufacturer's instructions before being autoclaved. Sterilised molten agar (15 mL) was then decanted into individual sterile petri dishes in a laminar flow cabinet. A prepared culture (section 2.5.3) of the chosen bacterium was diluted to a concentration of 10^7 CFUmL^{-1} in sterile PBS. A 100 µL aliquot of this suspension was pipetted onto the agar before being spread evenly over the surface. Then 30 µL of crude extract (15 mg mL^{-1} in extraction solvent) was pipetted onto a sterile filter disc (6 mm, Whatman) and the solvent was allowed to evaporate.

As soon as the solvent evaporated, the disc was placed upside down onto the agar. The plates were then incubated at 37 °C for 16 h. The zone of inhibition was measured in two directions (orthogonal to one another) and the size of zone was recorded minus the size of the filter disc (6 mm) (Figure 4-4).

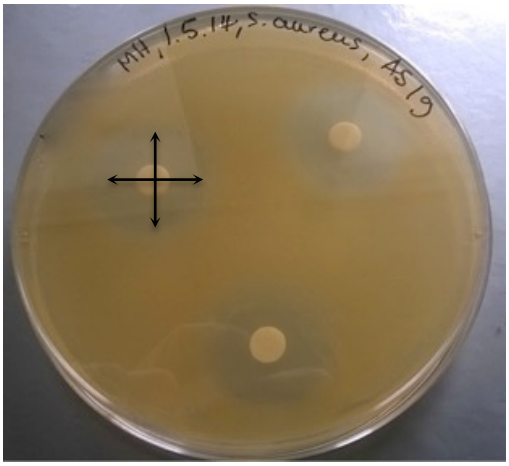


Figure 4-4 Digital image of an example of an MHA agar plate with MSSA growth and zones of inhibition around filter discs inoculated with 30 µL of the acetone extract of Agelas oroides (As19) (15 mg mL^{-1} in extraction solvent).

4.3.4 Microdilution method to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

The MIC assay was used to quantify any activity visualised by the bioautographic assay. Müller Hinton (MH) broth was prepared according to the manufacturer's instruction and autoclaved. An aliquot of 100 µL of MH broth was pipetted into a 96-well-plate using a multichannel pipette (wells 1-12). Then 100 µL of each test sample (concentration of 5.12 mg mL^{-1}) was added to the first well and mixed gently. To create a serial dilution, 100 µL from

the first well was removed and transferred into the second well and mixed gently. This procedure continued to the tenth well, where 100 µL of the solution was removed and discarded to achieve an equal volume of liquid in each well (Wiegand *et al*. 2008). Vancomycin was used as a positive control in each experiment also at an initial concentration of 5.12 mg mL^{-1}.

The bacteria used for MIC testing were MRSA (NCTC 11939) and *E. Coli* (NCIMB 12210) and were prepared, as section 2.5.3, before being diluted to 10^6 $CFUmL^{-1}$. 10 µL of this suspension was then added to the first 11 wells and mixed gently. The eleventh and twelfth wells served as controls, with well 11 containing broth and bacteria (positive control) and well 12 containing only broth (negative control).

A breathable microtitre membrane was applied onto the top of the plate to prevent it from drying out (Breathe-easy sealing membrane, Sigma-Aldrich, UK) The 96-well-plate was then incubated for 24 h on a shaking incubator at 100 rpm and 37 °C (Microspec, UK). To determine bacterial growth in the wells, the absorption was measured using an Elisa plate reader (Tecan 7 in nite 200pro, Tecan, Switzerland) at 595 nm. Confirmation of active bacteria was also visualised by staining each well with 5 µL of 2 mg mL^{-1} INT and incubating at 37 °C for 4 h. The MIC was deemed as the lowest concentration, which had considerably lower absorption than the positive control and showed no visible colour change from the INT stain (Figure 4-5).

Minimum bactericidal concentration (MBC) determination was determined by removing 20 µL of solution from each well and inoculating it onto Müller Hinton agar (in triplicate) before being incubated at 37 °C for 16 h. The concentration that did not show any growth on agar after incubation was recorded as the MBC (Figure 4-6).

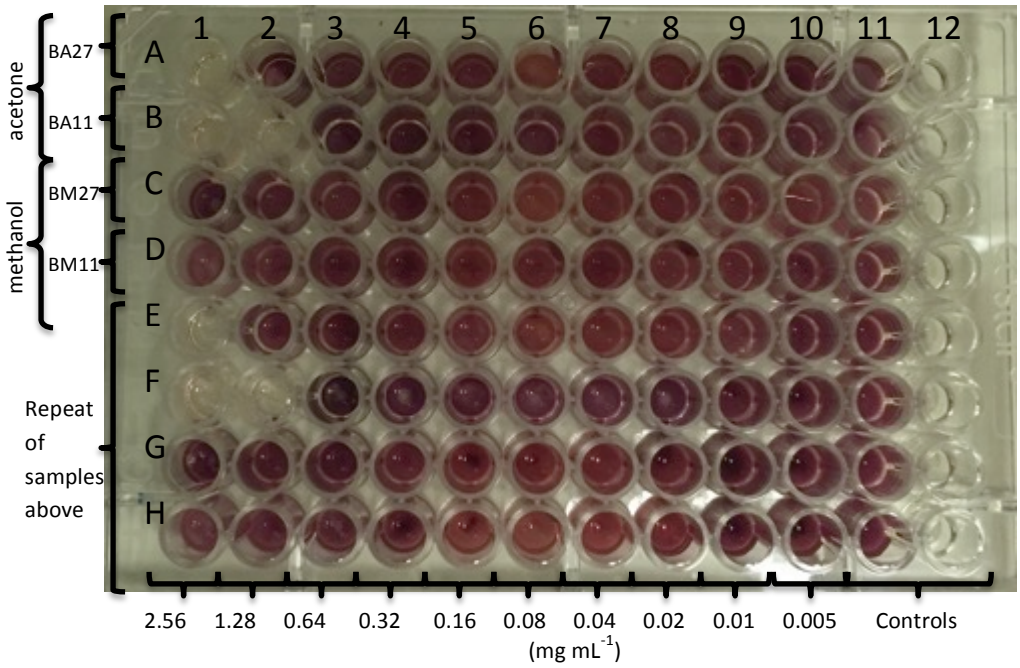


Figure 4-5 Digital image of an example of determination of MIC from sponge extracts against MRSA, using a 96 well plate and stained with INT to confirm bacterial growth. In this example no growth is observed in A1 and its repeat E1 (labelled), which was inoculated with MRSA exposed to a bacterial extract Ba27 (labelled) at a concentration of 2.56 mg mL^{-1} for 16 h. Thus the MIC of the acetone extract Ba27 (*Streptomyces violascens* strain G8A-22) was 2.56 mg mL^{-1}. Using the same method of determination B2 and its repeat F2 (*Exiguobacterium marinum* strain Tf-80) had an MIC of 1.28 mg mL^{-1}. Extracts (C) and (D), the methanol extract of the same strains labelled (BM27 and BM11) showed no inhibition of MRSA at the concentrations tested so their MIC was recorded as 5.12mg mL^{-1} (double concentration of the first well). The results are validated by the growth of bacteria in column 11 (+) and no growth in column 12 (-).

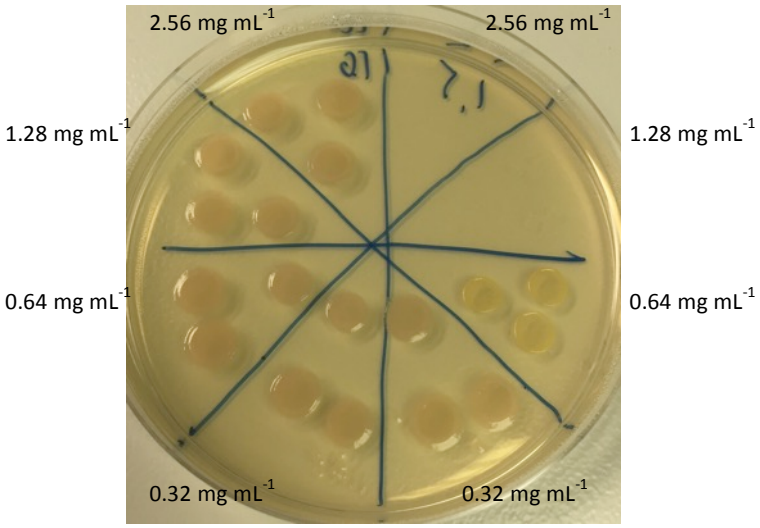


Figure 4-6 Digital image of the determination of the MBC of bacterial extract Ba27 (acetone extract of *Streptomyces violascens* strain G8A-22) and Ba11 (acetone extract of *Exiguobacterium marinum* strain Tf-80) against MRSA. In this example no growth was observed in the segment labelled 1.28mg mL^{-1} of sample Ba11 , which was inoculated with MRSA exposed to sponge extract at a concentration of 1.28 mg mL^{-1} (labelled) for 16 h. The MBC of sponge extract Ba11 was therefore 1.28 mg mL^{-1}. Ba27 displayed no ability to kill bacteria therefore its MBC was recorded as 5.12mg mL^{-1} (double the concentration of the first well).

4.4 Results and discussion

4.4.1 Targeted collection of Greek marine sponges

After facilitating authorisation for collection of samples (Appendix I), Archipelagos Institute of Marine Conservation (AIMC) produced a summary of sponges prevalent at the proposed collection sites in Samos and Fourni. A literature search was completed on this summary, analysing whether the sponge had been chemically characterised or tested for potential pharmaceutical activity. Each research paper classed as attempting to chemically characterise a sponge was considered as a 'match' (Table 4-1) if a sponge had more than 10 'matches' it was eliminated from the collection list. Sponges were also eliminated if they were deemed uncollectable, for example, encrusting and boring sponges, as they would be difficult to remove from rocks with a dive knife. Certain encrusting species and boring species were considered if they had more fleshy surfaces that may be scraped off a rock surface with a knife.

Table 4-1 Greek collection list. (N° of matches = number of research papers classed as attempting to chemically characterise a sponge. Search completed on Web of Knowledge up to and including 2012 including all databases. The following string search was employed ('Species name' and 'compound' or 'metabolite'). Matches with no limitations = 'Species name' matches.

Species name	N° of matches
Anchinoe roemeri	0 (1 match with no limitations)
Tethya aurantium	7
Spirastrella cunctatrix	0 (5 matches with no limitations)
Hemimycale columella	3

4.4.1.1 Collection of Greek sponge samples

Two of the four species identified in the collection list *Spirastrella cunctatrix* and *Hemimycale columella*, were collected from the Aegean Sea (Figure 4-7) surrounding Samos and Fourni.

(A)

(B)

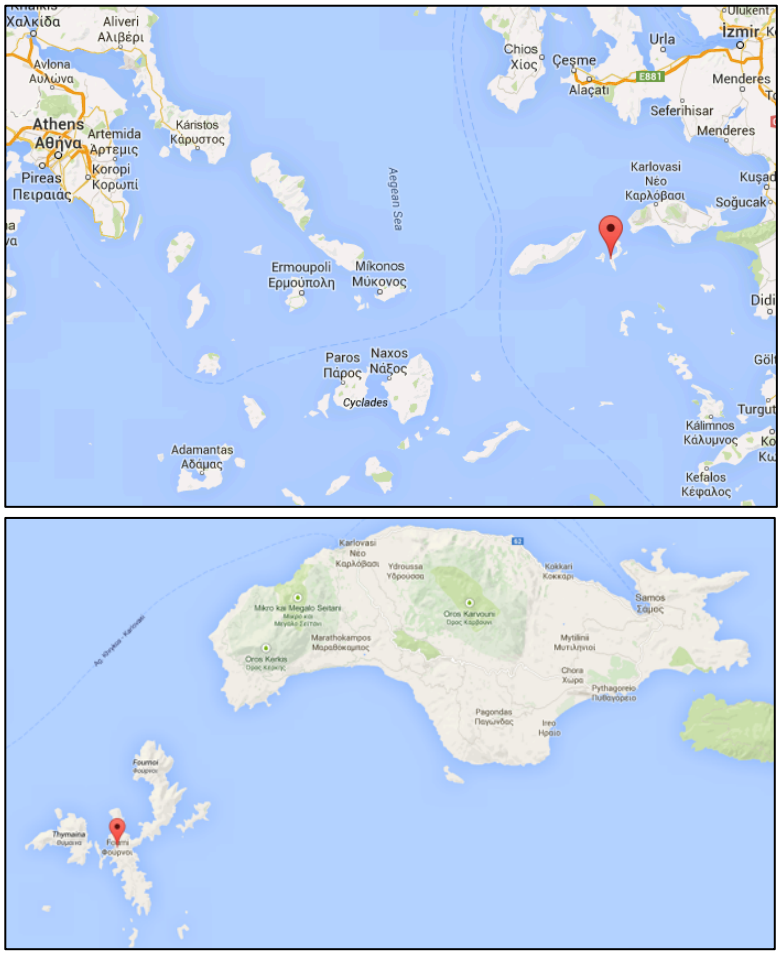


Figure 4-7 A map illustrating the geographical location of sponge samples collected, Fourni is highlighted with a pin drop (red symbol) in both images. (A): Aegean Sea spanning between the coasts of Greece (West) and Turkey (East); (B) Samos and Fourni. Images taken from 'Fourni, Greece.' Google Maps, Google, accessed at: https://www.google.co.uk/maps/place/Fournoi/@37.6433778,26.6299525,10z/data=!4m2!3m1!1s0x14bc5408a097b33f:0x49d13a8c7c965d56 **; date accessed 15th of March 2015.**

Other abundant species were also collected, to allow comparison of the environment in which they were found. Samples were collected via snorkelling and all species were collected between the depths of 0 to 10 m below sea level, at an average water temperature of 21 °C. Only up to half of a sponge was ever collected to ensure the environmental sustainability of this project. All samples were collected in plastic bags and labelled with the location and the depth where the sponge was found. A digital photograph was taken of each sponge *in situ*, as a record of the environment in which it was found, and a digital photograph was also captured next to the labelled bag on the surface before the sample was dried on the roof. Once the samples were completely dry, they were bagged and transported to the UK with the permission of DEFRA and with an export license from Greece (Appendix I).

4.4.1.2 Collection of other species

Some common and well-studied species that were not on the collection list were also collected enabling a comparison of the effect of environment on the compounds produced by a sponge. This may help determine the reason behind the production of any novel compounds in comparison to those suggested such as defence against predators (Proksch 1994) and prevention of fouling (Taylor *et al*. 2007). A further reason proposed for the production of unusual but active compounds is a competitive response to other species (Fattorusso *et al*. 2012). To investigate this hypothesis, some samples were taken from a competitive environment where multiple sponge species were present (A, Figure 4-8) and some were taken from an isolated environment (B, Figure 4.8). All the sponge species collected are displayed in (Table 4-2 Table 4-3 and Table 4-4)

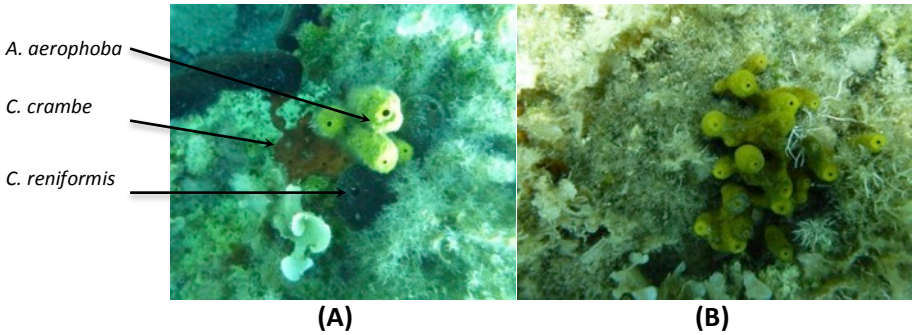
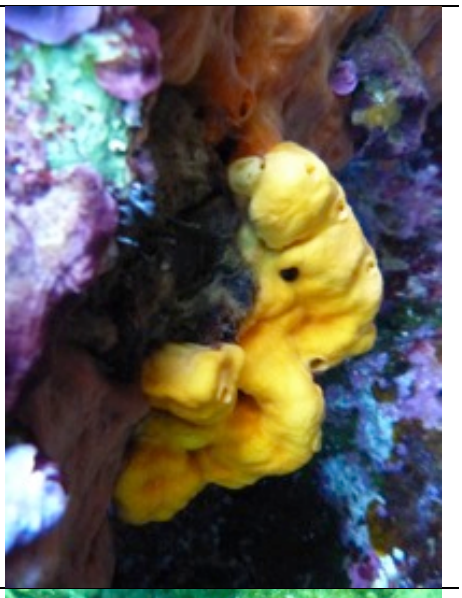
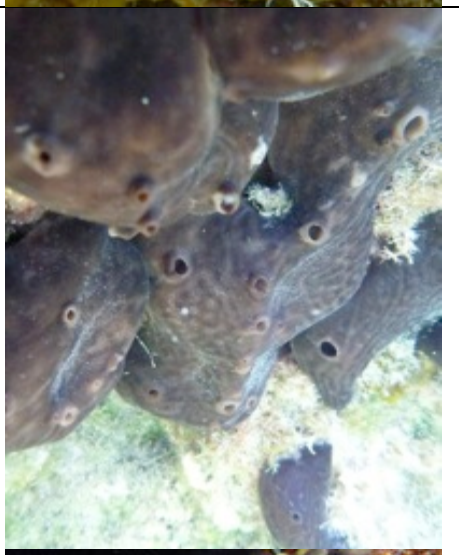
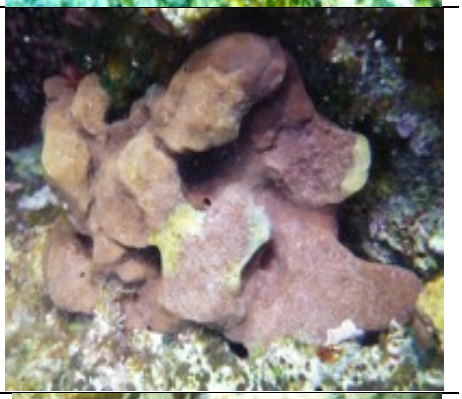


Figure 4-8 (A) *Aplysina aerophoba* (yellow tubular sponge) surrounded by other sponge species (*Crambe crambe* and *Chondrosia reniformis*). (B) *Aplysina aerophoba* in isolated conditions.

Table 4-2 Greek collected species list, including species name, location where the sample was collected, and pictures of a sample taken in *situ*.

Species Name	Site appearance	Description	Photographs
Agelas oroides	Samos, Fourni. Found in low light conditions, under rocks and in caves. Changes colour on ascent to surface from bright yellow to orange	Yellow, orange, red with an elastic texture. Massive with irregular lobes. Inhabits rock walls and caves.	
Aplysina aerophoba	Samos, Fourni. More commonly found at sites on Samos. Colour visibly deteriorates on surface.	Yellow and green fleshy mounds with thick chimneys. Chimneys are joined at the base and have a hole on the top end, growing up to 8 cm in height.	
Chondrilla nucula	Samos, Fourni. More prevalent at sites on Samos. Commonly found in shallow water in large collections.	Orange/brown giving the appearance with resemblance of potatoes. Form small colonies on hard rock, very common in the Mediterranean.	

			
Chondrosia reniformis	Samos, Fourni. Found in large numbers at differing depths. Often found next to other species, e.g. *Crambe crambe*	Marbled black and white. Smooth, velvet appearance shaped like a kidney.	 
Crambe crambe	Samos, Fourni. Prevalent in most locations in direct sunlight at shallow depths (<3m).	Thin encrusting layers of red and orange. Soft, irregular surface with multiple oscula giving the appearance with resemblance of veins. Colonies can grow large.	

			
Hemimycale columella	Samos, Fourni. Found in patches on rocks in direct sunlight.	Dark red to salmon encrusting with multiple small craters on the surface. Thick and soft appearance, resembling honeycomb.	
Petrosia ficiformis	Samos, Fourni	Purple and brown, but white in the absence of light. Hard and rough appearance with many spherical oscula. Inhabits rock overhangs and caves.	
Sarcotragus sp.	Samos, Fourni. Found singularly in open water in sunlight.	Large, black, massive structure sticking out of a rock with size of more than 50 cm in length. Rough texture with multiple holes across its surface.	 

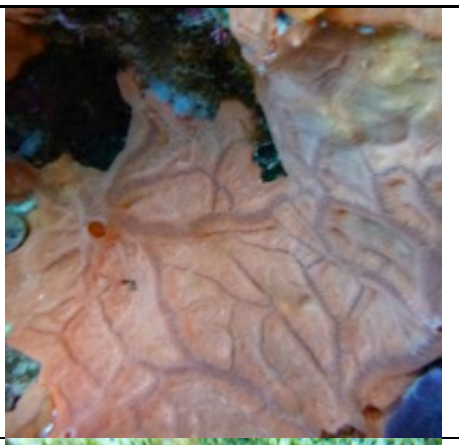
Spirastrella cunctatrix	Samos, Fourni. Found in caves and under rocks away from sunlight.	Red and orange encrusting, resembling *C. crambe* but it has a rougher texture with less oscula and more veins. Inhabits the underside of rocks.	
Ircinia variabilis	Fourni	The colour ranges between violet, pink, green and white with encrusting, massive or ramified, a rough appearance and few oscula.	

Table 4-3 Summary of sponge samples collected from Samos with assigned species ID number, date of collection and dry weight.

Sample ID	Species	Date of collection	Dry Weight (g)
Church Beach			
S1.	*Chondrosia reniformis*	4/9/12	10.534
S2.	*Sarcotragus sp. -*	4/9/12	24.140 + 19.990
S3.	*Chondrilla nucula.*	6/9/12	2.200
S4.	*Petrosia ficiformis*	6/9/12	12.923
S5.	*Chondrilla nucula*	12/9/12	1.788
S6.	*Chondrilla nucula*	12/9/12	7.593
S7.	*Sarcotragus sp.*	12/9/12	5.020
Gagou Beach (Vathy)			
S8.	*Chondrosia reniformis*	11/9/12	1.060
S9.	*Sarcotragus sp.*	11/9/12	6.750
S10.	*Crambe crambe*	11/9/12	19.076
S11.	*Sarcotragus sp.*	11/9/12	6.380
Glicorisa Beach			
S12.	*Crambe crambe*	5/9/12	1.908
S13.	*Aplysina aerophoba (isolated)*	5/9/12	3.040
S14.	*Petrosia ficiformis.*	5/9/12	3.870
S15.	*Sarcotragus sp.*	5/9/12	26.542
S16.	*Sarcotragus sp.*	5/9/12	21.761 + 20.727 + 16.077
Jetty Beach			
S21.	*Chondrosia reniformis*	5/9/12	3.910
S19.	*Agelas oroides*	5/9/12	11.680 + 12.124
S18.	*Aplysina aerophoba (competitive)*	5/9/12	6.496
Skyli Beach			
S20.	*Chondrilla nucula.*	5/9/12	7.731
S17.	*Chondrosia reniformis*	5/9/12	2.951

Table 4-4 Summary of sponge samples collected from Fourni with assigned species ID number, , date of collection and dry weight.

Sample ID	Species	Date of collection	Dry Weight (g)
Elidaki			
F1.	*Spirastrella cunctatrix*	10/9/12	2.121
F2.	*Chondrosia reniformis*	10/9/12	2.762
F3.	*Agelas oroides*	10/9/12	13.148
F4.	*Chondrosia reniformis*	10/9/12	
Vitsilia			
F5.	*Ircinia variabilis*	7/9/12	3.939
F6.	*Agelas oroides*	7/9/12	12.515
F7.	*Petrosia ficiformis*	10/9/12	6.101
F8.	*Petrosia ficiformis*	10/9/12	6.781
F9.	*Unknown*	10/9/12	
F10.	*Spirastrella cunctatrix*	10/9/12	12.158
Kampi			
F11.	*Ircinia variabilis*	7/9/12	2.499
F12.	*Sarcotragus sp.*	7/9/12	31.227
Ag. Giannis			
F13.	*Hemimycale columella*	7/9/12	6.491
F14.	*Agelas oroides*	7/9/12	4.678
F15.	*Crambe crambe*	7/9/12	1.349
F16.	*Crambe crambe*	7/9/12	2.557
F17.	*Petrosia ficiformis*	7/9/12	4.694
F18.	*Sarcotragus sp*.	7/9/12	14.202
Petrakopio			
F19.	*Crambe crambe*	8/9/12	7.060

4.4.2 Targeted collection of Welsh marine sponge samples

The collectable list of Welsh sponges was developed in the same manner as those from Greece (section 4.4.1), except the original list of abundant samples in the area was compiled by Claire Goodwin and the research team from Skomer (section 4.1.1.1). Each research paper classed as attempting to chemically characterise a sponge was considered as a ‘match’ (Table 4-5), if a sponge had more than 10 ‘matches’ it was eliminated from the collection list.

Table 4-5 Welsh collectable species list. N° of matches = number of research papers classed as attempting to chemically characterise a sponge. Search completed on Web of Knowledge up to and including 2012 including all databases. The following string search was employed ('Species name' and 'compound' or 'metabolite'). Matches with no limitations = 'Species name' matches.

Species name	N° of matches on Web of Knowledge
Halichondria panicea	10
Suberites ficus	1
Amphilectus fucorum	0 (6 matches with no limitations)
Hemimycale columella	3
Tethya citrina	0 (19 matches with no limitations)
Dysidea fragilis	7
Hymeniacidon perleve	10

Table 4-5 shows the collectable list of sponge species developed with local experts and the number of matches found when looking at how much secondary metabolite research had been completed on each species. On closer examination of the actual results, there were some interesting points. *Hemimycale columella* could be found in both Greek and Welsh waters so if any material could be collected this would make an interesting comparison. Also, although it appears *Halichondria panicea* had comparatively high number of matches examining its secondary metabolites. On closer examination of each of these matches, the majority of studies were analysing other aspects of a sponge such as the presence of bacteria (Imhoff *et al*. 2011; Lang *et al*. 2004; Lee *et al*. 2001; Wicke *et al*. 2000), the biology/ecology of the sponge genus (Krasokhin and Wijffels 2008), a review article (Belarbi *et al*. 2003) and an article that mentioned the sponge in reference (Casapullo *et al*. 1993). Only two matches described direct mining of sponge material for compounds (Toth and Lindeborg 2008; Christophersen *et al*. 1989). Both the commensal bacteria and the natural products produced by *Hymeniacidon perleve and Halichondria panicea* have been well studied (Table 4-5), which could make an interesting comparison for the materials extracted from a sponge and their origins.

4.4.2.1 Targeted collection of Welsh sponge samples via SCUBA from Milford Haven Waterway

Three sponge samples were collected by SCUBA on the 4th August 2013 in the Milford Haven Waterway, Angle Bay-Landing craft wreck, with an average water temperature of 13 °C. Although three different sponge species samples were collected (Table 4-6), due to low visibility, only the digital photograph of *Amphilectus fucurom in situ* (Figure 4-9) appeared

clearly. Samples were kept chilled, in a polystyrene box, in sealed bags and transported to the laboratory.

Table 4-6 Collection summary of sponge samples collected from Milford Haven Waterway.

Species ID	Sponge species	Dry weight (g)
j15	*Suberites ficus*	1.487g
j14	*Halichondria panicea*	0.446g
j02	*Amphilectus fucurom*	1.336g

Figure 4-9 A digital image of *Amphilectus fucurom* (j02) *in situ* found at a depth of approximately 25m in Milford Haven Waterway.

4.4.2.2 Processing samples

Samples were washed in the laboratory before being split into two. Samples for chemical testing were frozen immediately to −80 °C and samples reserved for bacterial testing were suspended in 10 % DMSO with sterile seawater before being frozen to −80 °C. Samples for chemical testing were processed (2.2.2) and then extracted using the sequential extraction method (2.2.3), forming three extracts per sample.

4.4.2.3 Collection of samples during low spring tide at Pembroke estuary.

Sponge samples were collected from close proximity to the Cleddau Bridge, Pembrokeshire (Figure 4-10) at a particularly low Spring tide on the 21st of August 2013. During this exceptionally low tide, areas of the seabed, which are normally never exposed to air were uncovered, revealing many different sponge species that are usually only collectable by

SCUBA. No more than half of a sponge sample was ever removed to ensure the sustainability of this project.

Figure 4-10 A map illustrating the geographical location of area of collection near Cleddau Bridge, Pembrokeshire. Images taken from 'Cleddau Bridge, Pembroke Dock.' Google Maps, Google, accessed at : https://www.google.co.uk/maps/place/Cleddau+Bridge+Hotel/@51.700764,-4.932721,13z/data=!4m2!3m1!1s0x486930f35d2f8a89:0xd8f27d44ec0fc4c8 , date accessed 15th October 2015

A digital photograph of each sample was taken *in situ* to give an idea of the conditions where it was found, particularly whether it was in a competitive environment. All sponges were identified by Jennifer Jones and Claire Goodwin (Department of Natural Sciences, National Museums Northern Ireland). Some samples showed unusual polymorphisms and their spicule formation had to be analysed microscopically to confirm identification. Sample 21 and 22 (Figure 4-11) showed unusual shapes and textures for *Halichondria panicea* but as a species, it is known to be very polymorphic (Christophersen *et al*. 1989). All collected samples were processed in the same manner as section 4.4.2.2.

1. *Hymeniacidon perleve.*

2. *Hymeniacidon perleve.*

3. *Hymeniacidon perleve.*

4. *Hymeniacidon perleve.*

5. *Hymeniacidon perleve.*

6. *Hymeniacidon perleve.*

7. *Hymeniacidon perleve.*

8. *Hymeniacidon perleve.*

9. *Amphilectus fucorum.*

11. *Halichondria panicea*

12. *Halichondria panicea*

13. *Hymeniacidon perleve.*

14. *Halichondria panicea*

15. *Halichondria panicea.*

16. *Halichondria panicea*

18. *Halichondria panicea*

19. *Halichondria panicea*

20. *Dysidea fragilis*

21. *Halichondria panicea*

22. *Halichondria panicea*

23. *Halichondria panicea*

24. *Haliclona oculata*

25. *Halichondria panicea*

26. *Halichondria panicea*

27. *Halichondria panicea*

Figure 4-11 Digital images of samples collected from Pembroke estuary. Samples 10 and 17 are not included as they were samples of plant material collected for use as standards for comparison.

4.4.3 Chemical analysis of Greek sponge samples

In total 39 samples were collected from Greece, 21 from Samos and 18 from Fourni. Nine different species were sampled including two from the collection list and other species were particularly targeted for the varying conditions in which they could be found. Extraction was performed using the sequential extraction method (section 2.2.2) with some minor changes (section 4.3.1). Due to the larger number of samples, the method previously used was adapted by scaling it down, using less initial material and less solvent. This was done to ensure sufficient material was extracted to complete the bioautographic overlay activity assay in order to determine lead compounds to carry forward, and then discard extracts that showed no activity.

The nomenclature for the Greek samples was based on the information listed in Table 4-7. The samples were identified by the Sample ID listed in Table 4-7 and preceded by the first letter of the solvent used for extraction. For example, the acetone extract of sample f4 (*Chondrosia reniformis* collected from Fourni) was labelled Af4. Any further separations of the extract were named by adding a number to name assigned e.g. fraction Af4.1.

Table 4-7 Extraction summary of collected species of Greek sponge. Extraction was performed on 0.5g of material from each sponge sample. ± = Standard error of mean.

Sample ID	Dry collected weight (g)	Hexane extract (g)	Acetone extract (g)	Methanol extract (g)	Total mass (g)
***Chondrosia reniformis* (Cr)**					
f4	5.122	0.120	0.006	0.058	0.184
s1	10.534	0.115	0.015	0.070	0.200
s8	1.060	0.089	0.008	0.051	0.148
s17	2.951	0.113	0.002	0.065	0.180
S21	3.910	0.111	0.010	0.063	0.184
f2	2.762	0.113	0.004	0.050	0.167
Mean	4.390	0.110±0.04	0.007±0.002	0.059±0.003	0.177±0.009
***Sarcotragus sp.* (Ss)**					
s2	44.130	0.135	0.012	0.073	0.220
s7	5.020	0.112	0.013	0.083	0.208
s9	6.750	0.112	0.012	0.062	0.186
s11	6.380	0.132	0.017	0.073	0.222
s15	26.542	0.136	0.015	0.070	0.221
s16	58.565	0.139	0.013	0.077	0.229
f12	31.227	0.105	0.020	0.074	0.199
f18	14.202	0.131	0.015	0.082	0.228
Mean	24.102	0.125±0.005	0.015±0.001	0.074±0.002	0.214±0.008
***Chondrilla nucula* (Cn)**					
s3	2.200	0.146	0.005	0.068	0.219
s5	1.788	0.140	0.008	0.069	0.217
s6	7.593	0.115	0.009	0.041	0.165
s20	7.731	0.114	0.011	0.066	0.191
Mean	4.828	0.129±0.008	0.008±0.001	0.061±0.007	0.198±0.0016
***Crambe crambe* (Cc)**					
s10	19.076	0.153	0.030	0.050	0.233
s12	1.908	0.137	0.100	0.019	0.166
f15	1.349	0.144	0.013	0.078	0.235
f16	2.559	0.162	0.025	0.097	0.284
f19	7.060	0.196	0.026	0.073	0.295
Mean	6.390	0.158±0.010	0.046±0.004	0.063±0.013	0.267±0.028
***Aplysina aerophoba* (Aa)**					
s13	3.040	0.096	0.029	0.117	0.242
s18	6.496	0.116	0.021	0.130	0.267
Mean	4.768	0.106±0.010	0.025±0.004	0.124±0.006	0.254±0.020
***Petrosia ficiformis* (Pf)**					
s4	12.923	0.105	0.010	0.070	0.185
s14	3.870	0.061	0.011	0.060	0.132
f7	6.131	0.114	0.009	0.031	0.154
f8	6.781	0.082	0.008	0.034	0.124
f17	4.694	0.118	0.014	0.061	0.193

Sample ID	Dry collected weight (g)	Hexane extract (g)	Acetone extract (g)	Methanol extract (g)	Total mass (g)
Mean	6.880	0.084±0.011	0.010±0.001	0.051±0.008	0.145±0.020
***Spirastrella cunctatrix* (Sc)**					
f1	2.121	0.077	0.008	0.032	0.117
f10	12.158	0.076	0.007	0.026	0.109
Mean	7.140	0.076±0.011	0.007±0.000	0.029±0.003	0.113±0.004
***Hemimycale columella* (Hc)**					
f13	6.491	0.128	0.008	0.021	0.157
***Agelas oroides* (Ao)**					
s19	23.804	0.110	0.013	0.074	0.197
f3	13.148	0.104	0.044	0.091	0.239
f6	12.515	0.108	0.011	0.084	0.203
f14	4.678	0.102	0.061	0.079	0.242
Mean	13.536	0.106±0.002	0.032±0.012	0.082±0.004	0.220±0.018
***Ircinia variabilis* (Iv)**					
f5	3.939	0.091	0.009	0.093	0.193
f11	2.499	0.091	0.013	0.093	0.197
Mean	3.219	0.091±0.000	0.011±0.002	0.093±0.000	0.195±0.002

Once the extracts were dried and weighed, some clear species differences became apparent. The yield % (w/w) recovered varied significantly between species, with *Aplysina aerophoba* and *Crambe crambe* providing approximately 50 % yield but species such as *Spirastrella cunctatrix* providing approximately 20 % yield (Figure 4-12). The differences in yield between samples were compared using the Kruskal Wallis test (section 2.6.2.2), which completes a pairwise comparison between groups. This showed a significant difference ($p > 0.05$) between various sponge species with the largest difference observed between *Spirastrella cunctatrix* and *Crambe crambe*.

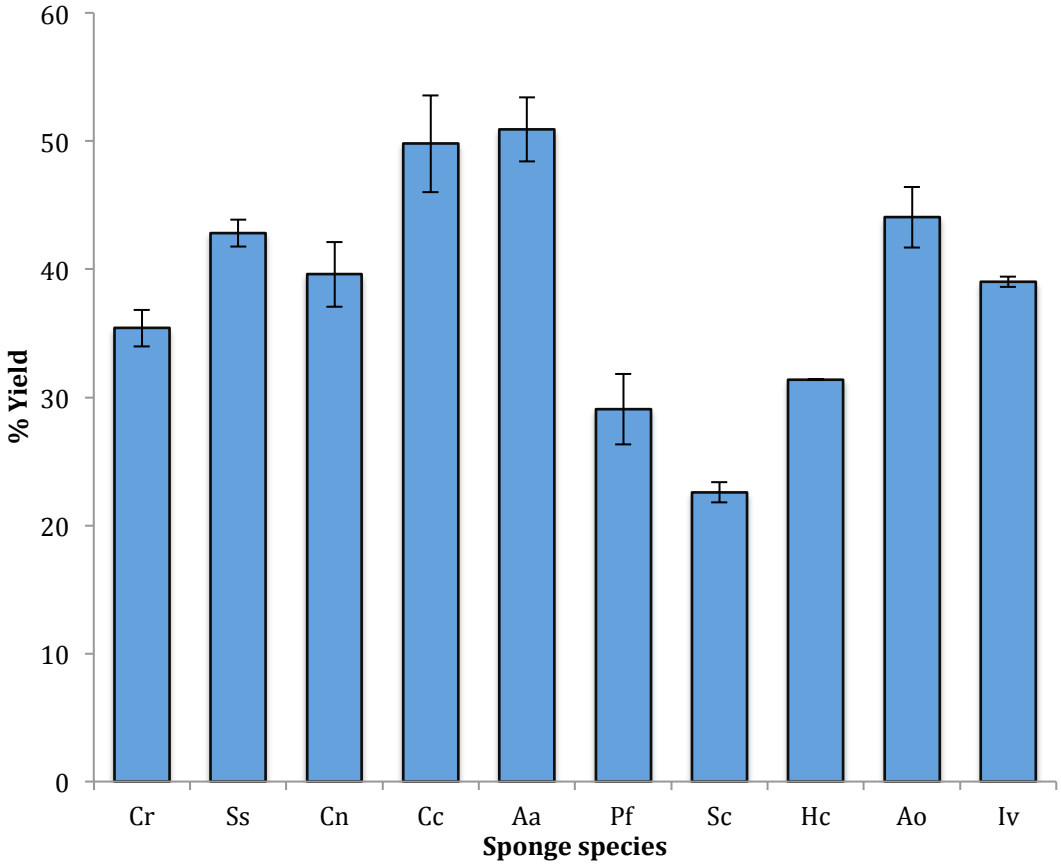


Figure 4-12 Percentage (%) yield of Greek sponge species. Error bars represent standard error of mean. Species abbreviations and number of samples (n): Cr = *C. nucula* (n = 6), Ss = *Sarcotragus sp*. (n = 8), Cn = *C. nucula* (n = 4), Cc = *C. Crambe* (n = 5), Aa = *A. aerophoba* (n = 2), Pf = P*. ficiformis* (n = 5), Sc = *S. cunctatrix* (n = 2), Hc = *H. columella* (n = 1), Ao = *A. oroides* (n = 4) and Iv = *I. variabilis* (n = 2).

Interestingly, there was a notable visual difference between *Crambe crambe* and *Spirastrella cunctatrix* upon drying. Even though they looked extremely similar *in situ* (both bright orange encrusting sponges (Table 4-2)) and were very difficult to tell apart; once dried it was notable that *Crambe crambe* kept its colour whilst *Spirastrella cunctatrix* changed to a light brown or beige colour. One possible explanation could be that volatile pigments may have escaped from *Spirastrella cunctatrix* during the drying process in Greece. Another possible explanation could be that the *Spirastrella* samples were damaged by sunlight while being exposed to light during drying outdoors. *Spirastrella* was only found in very dark caves with no contact of direct sunlight whereas *Crambe crambe* was found abundantly in areas where sunlight could reach. Therefore, it is possible the presence of sunlight affected the stability of photosensitive pigments in *Spirastrella cunctatrix* samples. A further possible explanation could be that as both species were encrusting sponges and were difficult to remove from rocks, they often had parts of rock and shell embedded in them. Although great effort was

put in to removal of these foreign bodies, it is possible that these potentially added to the initial mass recorded, therefore reducing the relative yield obtained. Furthermore, there were also possibilities that some sponge samples were not evenly ground or that the sponge samples were made up from different constituents that were impossible to extract using the methods employed in this study. A difference was also observed when a direct comparison of yields was made to the species from the preliminary experiments in chapter 3; *A. oroides* (23 %) and *A. aerophoba* (41 %) compared to an average of 41 % and 51 % respectively from this multiple species study. It is likely these increased figures are due to the addition of a sonication step to the extraction process. Furthermore these comparisons demonstrate that *A. aerophoba* contains more extractable material than *A. oroides*, which was hypothesised in section 3.5.

The different sponge species were also extracted in varying proportions in the three main crude extraction solvents of different polarity (Figure 4-13), with over two thirds of yield from *Crambe crambe* extracted in hexane. This suggests that *Crambe crambe* is made up of a high proportion of non-polar compounds, unlike *Aplysina aerophoba,* which had 50 % of its total yield extracted in methanol, and therefore contained a higher proportion of polar compounds. This difference was compared using Kruskal Wallis, which showed a significant difference in the mass of hexane and methanol extracts of *Crambe crambe* and *Aplysina aerophoba* ($p < 0.05$). It was apparent that on average approximately 40 % yield was extracted from each sponge sample and over half of the yield was extracted in hexane. This was used to predict the quantity of material required for future extractions and analysis (Figure 4-13).

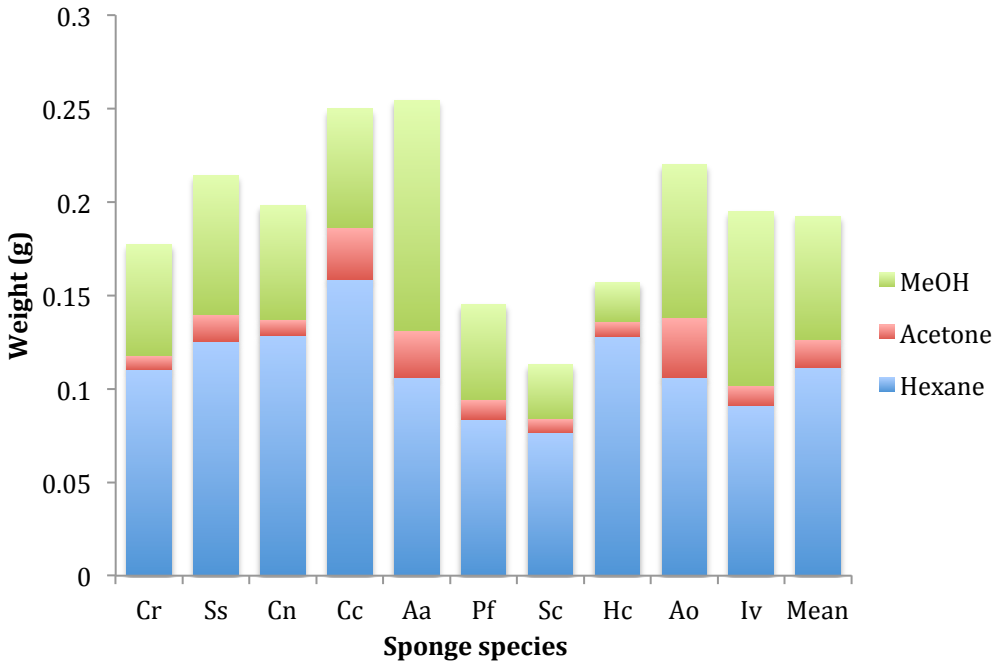


Figure 4-13 Average breakdown in total yield of compounds extracted in three main crude extraction solvents (methanol, acetone and hexane) used to extract various Greek sponge species samples. n = number of samples extracted per species. Species abbreviations and number of samples: Cr = *C. nucula*(n = 6), Ss = *Sarcotragus sp*.(n = 8), Cn = *C. nucula* (n = 4), Cc = *C. Crambe* (n = 5), Aa = *A. aerophoba* (n = 2), Pf = P. *ficiformis* (n = 5), Sc = *S. cunctatrix* (n = 2), Hc = *H. columella* (n = 1), Ao = *A. oroides* (n = 4) and Iv = *I. variabilis* (n = 2).

4.4.4 Chemical analysis of Welsh sponge samples

Unlike the Greek samples where only 0.5 g per sample was used for extraction, the whole of Welsh samples collected were used for extraction to avoid work repetition when more material was required. The disadvantage of this method was that it consumed large volumes of solvent compared to extraction of the Greek samples. It was decided to fully extract all dry Welsh samples as there were fewer specimens in comparison to the Greek collection (Table 4-8, Figure 4-14), this would also prevent the need for re-extraction if any leads were identified from the samples.

Table 4-8 Extraction summary of collected species of Welsh sponge. Extraction was performed with all material collected.

Sample ID	Collected weight (g)	(1) Hexane extract (g)	(2) Acetone extract (g)	(3) Methanol extract (g)
***Hymeniacidon perleve* (Hpe)**				
w1	9.05	0.169	0.069	0.524
w2	6.317	0.121	0.052	0.572
w345	10.375	0.173	0.056	1.154
w6	23.39	0.447	0.082	1.85
w7	8.524	0.159	0.046	0.919
w8	11.644	0.166	0.04	0.801
w16	1.233	0.017	0.008	0.112
Mean	10.076	0.179	0.050	0.847
Halichondria panicea (Hpa)				
w11	1.086	0.030	0.014	0.185
w12	1.334	0.034	0.017	0.180
w14	4.814	0.123	0.045	0.805
w15	4.225	0.125	0.045	0.708
w18	7.579	0.127	0.101	1.238
w19	7.322	0.132	0.082	1.201
w21	0.498	0.014	0.006	0.079
w22	8.757	0.160	0.100	1.370
w23	4.280	0.081	0.040	0.770
w25	1.940	0.054	0.015	0.299
w26	2.428	0.075	0.021	0.329
w27	2.779	0.088	0.022	0.364
j14	0.446	0.019	0.013	0.082
Amphilectus fucorum (Af)				
w9	3.176	0.058	0.051	0.385
j02	1.336	0.022	0.023	0.247
***Subertes ficus* (Sf)**				
j15	1.487	0.076	0.076	0.386
***Dysidea Fragilis* (Df)**				
w20	3.813	0.025	0.022	0.910
***Cliona celata* (Cc)**				
CC	4.124	0.186	0.008	1.011

The nomenclature for the Welsh samples was based on the information listed in Table 4-8. The samples were identified by the Sample ID listed in Table 4-8 and preceded by the number assigned to the solvent used for extraction. *E.g.* the acetone extract of sample w20 (Dysidea fragilis) was labelled 2w20. Any further separations of the extract where named by adding a number to name assigned e.g. 2w20.1. Samples from Greece and Wales were labelled differently to allow instant recognition between the samples.

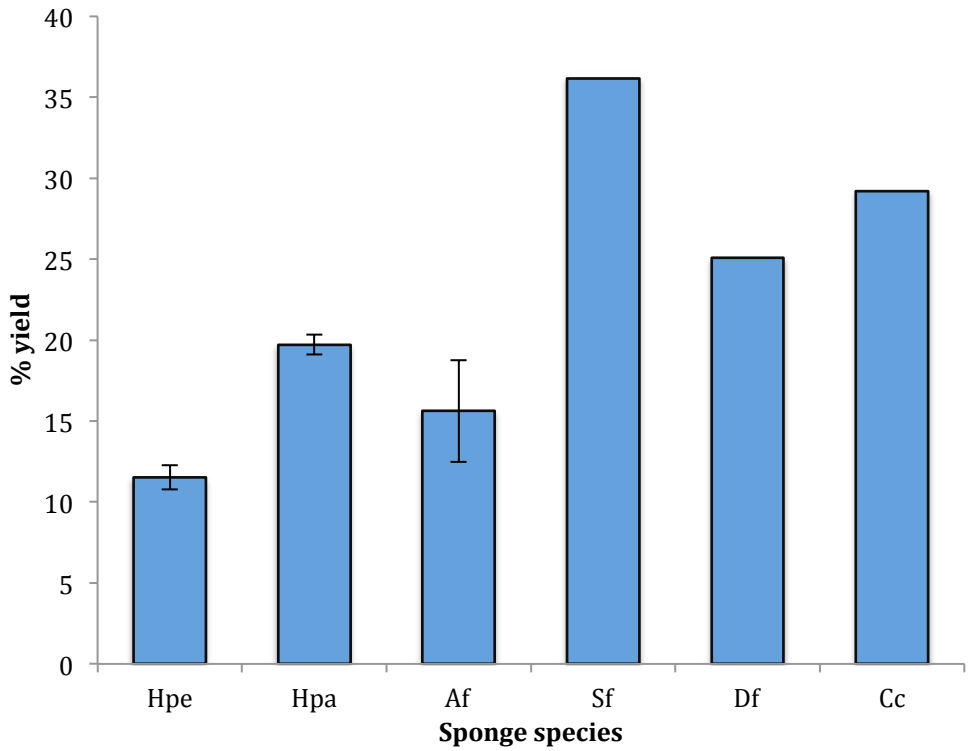


Figure 4-14 Percentage (%) yield of Welsh sponge species. Error bars represent standard error of mean. Species abbreviations and number of samples (n): Hpe = *H. perleve* (n = 7), Hpa = *H. panicea* (n = 13), Af = *A. furonum* (n = 2), Sf = *S. ficus* (n = 1), Df = *D. fragilis* (n = 1), Cc = C. celata (n = 1).

The average yield for Welsh samples was statistically significantly ($p < 0.05$) lower than that of the Greek samples, 18.49% ±1.290 (Standard error) and 39.65% ±1.389 (Standard error) respectively (Figure 4-15). There are a few possible explanations for this result. Firstly, Welsh samples may not contain as much 'extractable' material and may be made of more skeletal material such as spicules. Secondly, the majority of the Welsh samples collected where encrusting species, and similar to the Greek encrusting species, they often had foreign materials e.g. stones and shells embedded into them, which may not have been fully removed prior to extraction. This can be seen in Figure 4-11, where for example Welsh samples 1 to 3 were completely loose and formed around a piece of rock or shell.

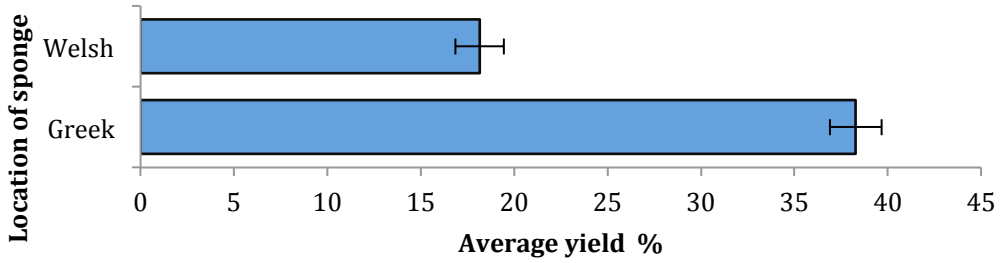


Figure 4-15 Comparison of percentage (%) yield of sponge species from Greece and Wales. Error bars represent standard error of mean. For Greek samples n= 38 and for welsh samples n = 25.

The extract constituents of each Welsh sponge samples were then compared to one another (Figure 4-16), which showed a significant difference in proportion of yields contained in different crude extracts compared to that found in the Greek samples. In the Welsh samples, it was notable that on average, larger proportions of total yield were extracted in methanol (Figure 4-13).

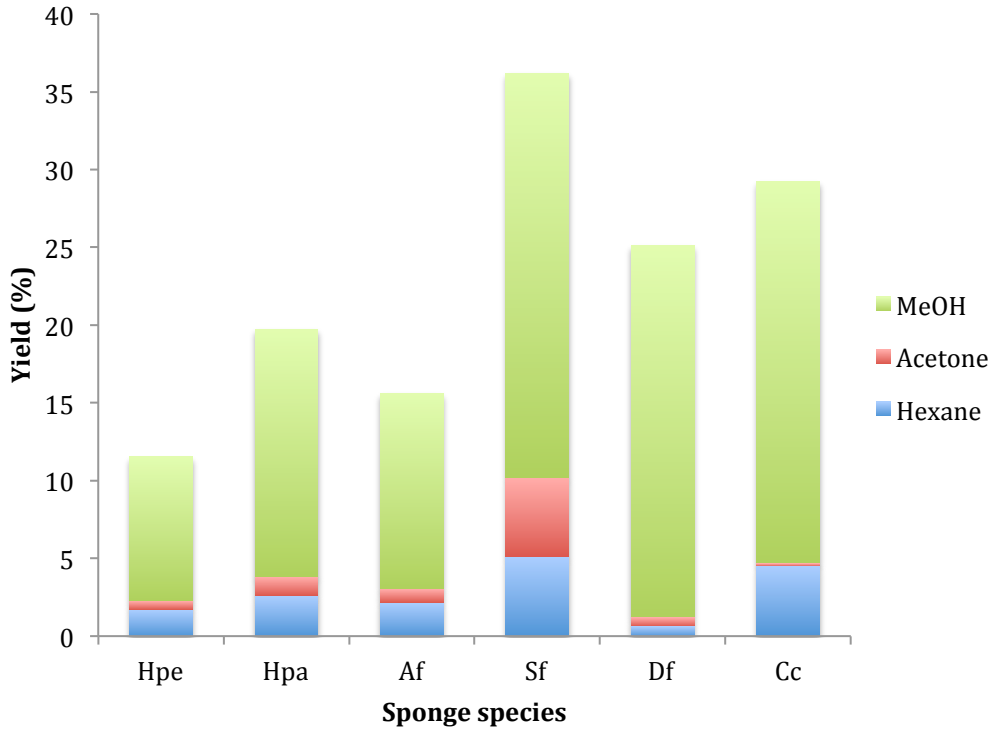


Figure 4-16 Average breakdown in total yield of compounds extracted in three main crude extraction solvents (methanol, acetone and hexane) used to extract various Welsh sponge species samples. n = number of samples extracted per species. Species abbreviations and number of samples (n): Hpe = *H. perleve* (n = 7), Hpa = *H. panicea* (n = 13), Af = *A. fucorum* (n = 2), Sf = *S. ficus* (n = 1), Df = *D. fragilis* (n = 1), Cc = C. celata (n = 1).

4.4.5 Comparison between the chemical composition of Greek and Welsh sponge samples

The differences observed in extract constituents of Greek and Welsh sponges were statistically compared. As the average yield was so different between Greek and Welsh sponges (Figure 4-15) a constituent-yield comparison similar to that completed for species from the same collection site (Figure 4-13, Figure 4-16) would provide little comparable information. For this reason the percentage composition of each component constituent was compared.

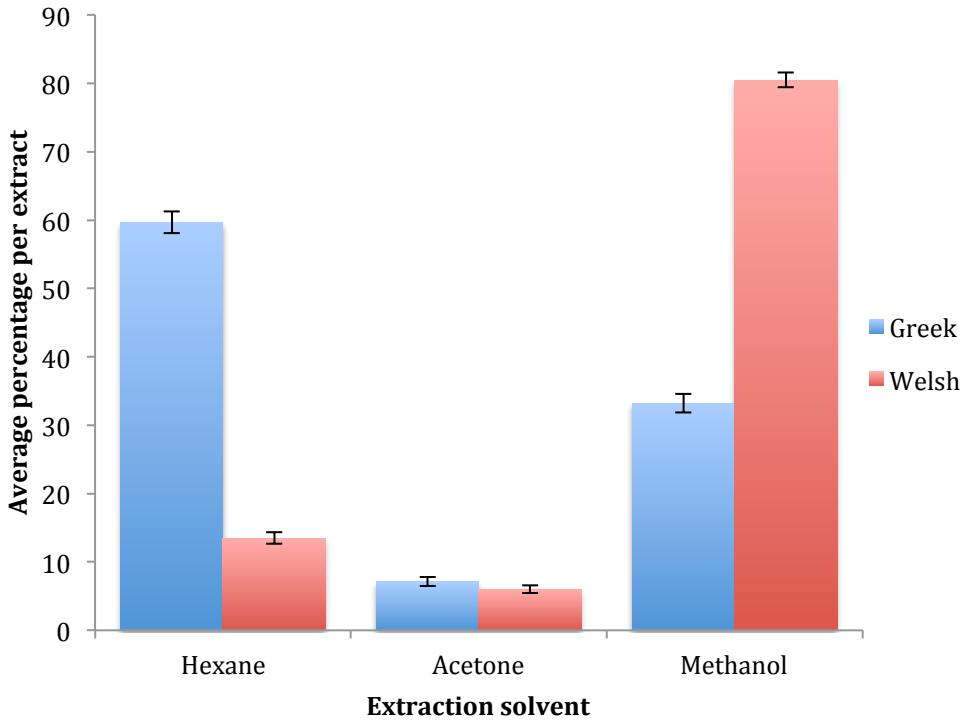


Figure 4-17 Comparison of the average percentage (%) of material extracted by each solvent between Greek and Welsh sponges. Error bars represent standard error of mean. For Greek samples n= 38 and for Welsh samples n = 25.

The largest proportion of extracted material from the Greek samples was found in the hexane extract whereas the largest proportion of extracted material from the Welsh samples was found in the methanol extract. This observed difference was statistically significant ($p < 0.05$). This could be explained by the fact that the Welsh sponges studied simply consisted of different proportions of compounds to their Greek counterparts. Another plausible explanation could be the difference in environment where the Welsh samples were found.

The Welsh samples were often covered in algae and a lot of sediment was present in the surrounding water, both of which could have formed a proportion of the material extracted.

4.4.6 Assessing the antibacterial activity of Welsh and Greek marine sponge extracts

4.4.6.1 Direct bioautographic chromatography as a method for assessing the antibacterial activity of marine sponge extracts

All samples were tested against both MSSA and E. *coli* and results were recorded as either positive or negative. If there was a clear zone of inhibition, it was recorded as extract with positive activity and if there was no inhibition, it was recorded as extract with negative result. Any antimicrobial activity was then matched against digital images of the TLC plate prior to the overlay method and active compounds could then be targeted for extraction and identification.

Although every effort was made to ensure the concentrations of each extract pipetted onto the plate was consistent, this method cannot be used to validate the intensity of inhibition, as the concentration of each separated compound within an extract and how far they spread on the plate was not controllable. For examples some extracts may contain predominantly one active compound which may spread over a large area of the plate due to its affinity for silica (indicating significant activity) or a similarly active compound may be resolved to a fine band (producing a seemingly smaller area of inhibition) whilst other extracts may contain multiple active compounds that do not resolve well on the plate, each would produce a different picture of activity on the overlaid stained plate. Essentially, an idea of antimicrobial activity and the location of compounds with activity may be gauged from the technique but the method is not quantifiable. Concentration dependent tests on fully separated compounds have to be performed to provide more accurate quantitative information such as the MIC of a compound.

In summary, 25 Welsh samples were tested (total of 75 extracts), and 39 Greek samples were tested (total of 117 extracts). Overall, 18 (24%) of Welsh extracts and 27 (23%) of Greek extracts showed activity against MSSA or *E. coli*, using the overlay assay. These results suggest that the Welsh extracts showed comparable activity towards MSSA to those from Greece. Activity was also demonstrated against *E. coli*, which is not always the case when a bacterium from a sponge is cultured (Abdelmohsen *et al*. 2010). Fewer extracts showed activity towards *E. coli* with only four extracts from Wales and ten extracts from Greece

active against *E. coli*, this was to be expected as similar picture has been seen in other studies (Rifai *et al*. 2005; Abbas *et al*. 2011).

Variation was observed in Rf correlated to activity, in different extracts, although some potentially common compounds overlapped between extracts. *E.g.* activity found at Rf 0.9 in all *A. oroides* samples (Table 4-9). The variety of Rf values of activity, from the same extract and across samples, was a useful result as it indicated that a range of antibacterial compounds were present in the samples thus providing good leads for subsequent separation and identification of active chemicals within extracts.

Table 4-9 Summary of antimicrobial activity determined using bioautographic technique. Rf values of eluted extracts (20mg ml^{-1}) with activity against MSSA. (+ = positive activity; - = no activity; Prefix of H = hexane extract, Prefix of A = acetone extract; M = methanol extract of Greek samples. Prefix of 1 = hexane extract, 2 = acetone extract; 3 = methanol extract of Welsh sample. Solvent system for hexane extracts 2:8 (acetone : hexane), for acetone extracts 4:6 (acetone : hexane) and for methanol extracts 1 : 9 (methanol dichloromethane). Species abbreviations: Hpe = *H. perleve,* Hpa = *H. panicea*, Af = *A. fucorum*, Sf = *S. ficus*, Df = *D. fragilis,* Cr = *C. nucula,* Ss = *Sarcotragus sp*, Cn = *C. nucula*, Cc = *C. Crambe*, Aa = *A. aerophoba*, Pf = P. *ficiformis*, Sc = *S. cunctatrix*, Hc = *H. columella,* Ao = *A. oroides* and Iv = *I. variabilis*. Vancomycin 20mg mL^{-1} was used as a positive control.

Greek ID	Extract	Species	Activity against			Welsh ID	Extract	Species	Activity against		
			MSSA	*E. coli*					*MSSA*	*E. coli*	
Hf4		Cr	+	-	0.7, 0.8	1j02		Af	+	-	0,0.1
As1		Cr	+	-	0-0.05, 0.8	2w6		Hpe	+	-	0.45
As6		Cn	+	-	0.8-1.0	2w8		Hpe	+	-	0.45
As10		Cc	+	+	0,1	2w9		Af	+	-	0.45
As12		Cc	+	-	0	2w14		Hpa	+	+	0-0.4
As13		Aa	+	+	0-0.1, 0.8-0.9	2w15		Hpa	+	+	0.4
As19		Ao	+	+	0-0.1, 1.0	2w16		Hpa	+	+	0
As20		Cn	+	-	0.9	2w18		Hpa	+	+	0.1-0.15,
Af1		Sc	+	-	0						0.2-0.25, 0.35
Af3		Ao	+	+	0-0.4	2w20		Df	+	+	0-0.1, 0.3
Af6		Ao	+	-	0, 0.1, 0.9-1.0	2j14		Hpa	+	-	0-0.05
Af11		Iv	+	-	0.9-1.0	2j15		Sf	+	-	0
Af13		Hc	+	-	0	2J02		Af	+	-	0
Af14		Ao	+	-	0,0.1,0.8-1.0	3w2		Hpe	+	-	0.4,0.6
Af17		Pf	+	-	0, 0.1, 0.8, 1.0	3w12		Hpa	+	-	0-0.1
Ms1		Cr	+	-	0.2	3w18		Hpa	+	-	0-0.1
Ms2		Ss	+	-	0.5	3w20		Df	+	-	0-0.1
Ms10		Cc	+	+	0-0.4	3w21		Hpa	+	-	0-0.2
Ms12		Cc	+	+	0.1, 0.3	3j14		Hpa	+	-	0-0.1
Ms13		Aa	+	+	0.8	3j15		Sf	+	-	0-0.6
Ms18		Aa	+	+	0.9	3j02		Af			0.1-0.3
Ms19		Ao	+	+	0.4, 0.9						
Mf3		Ao	+	-	0.3-0.4, 0.9						
Mf6		Ao	+	-	0.3,0.9						
Mf12		Ss	+	-	0.6						
Mf14		Ao	+	-	0.4,0.9						
Mf15		Cc	+	+	0-0.1, 0.25						
Mf16		Cc	+	-	0,0.1						
Mf19		Cc	+	+	0,0.5						

4.4.6.2 Disc diffusion assay as a method for assessing the antibacterial activity of marine sponge extracts against MSSA

The disc diffusion assay was performed for all acetone extracts from Greek and Welsh sponges and a single hexane extract from a Greek sponge against MSSA. This method allowed direct statistical comparison between extracts and their antibacterial activity. However, direct comparison between extracts when different solvent systems were used was not be feasible due to potential difference in diffusion coefficient across the agar of compounds. For this reason, this test was only completed on acetone extracts and one hexane extract that showed significant activity in the overlay assay (section 4.4.6.1).

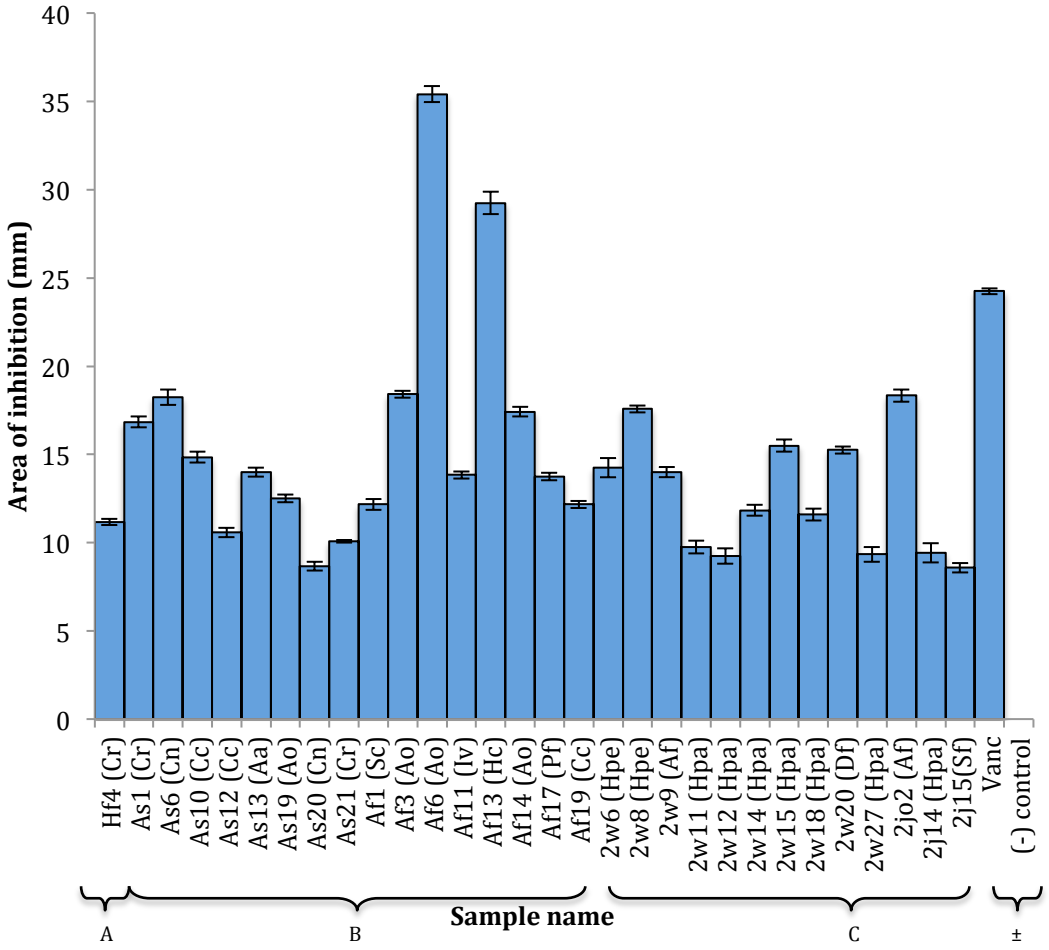


Figure 4-18 Area of inhibition of Greek and Welsh sponge sample extracts (15mg ml^{-1}) that were active against MSSA following disc diffusion assay. (Error bars = standard error; n=3). A = Hexane extract of Greek species. B = Acetone extracts of Greek species. C = Acetone extracts of Welsh species. (±) = Positive (Vancomycin 15mg ml^{-1}) and negative control. Species abbreviations: Hpe = *H. perleve* , Hpa = *H. panicea*, Af = *A. fucorum*, Sf = *S. ficus*, Df = *D. fragilis,* Cr = *C. reniformis*, Cn = *C. nucula*, Cc = *C. Crambe*, Aa = *A. aerophoba*, Pf = P. *ficiformis*, Sc = *S. cunctatrix*, Hc = *H. columella,* Ao = *A. oroides* and Iv = *I. variabilis*.

Due to the large number of samples tested, only extracts that displayed positive antibacterial property against MSSA are reported. The results showed the two most active extracts were Af6, *Agelas oroides,* and Af13, *Hemimycale columella*, ($p < 0.05$)(Figure 4-18), when compared to other extracts, they also showed significantly greater activity than Vancomycin, suggesting the compounds responsible for activity were very potent. When also compared to other research papers including those were individual compounds are tested these zones of inhibition are particularly large (Youssef *et al*. 2013). The results showed an almost perfect correlation with the bioautographic overlay method confirming the overlay method was effective at identifying antibacterial activity.

All results were compared using Kruskal Wallis test which showed that the 15 most active samples were As1, As6, As13, Af3, Af6, Af11, Af13 Af14, Af17, 2w6, 2w8, 2w9, 2w15, 2w20 and 2Jo2 ($p < 0.05$) when compared to the other extracts. These active species were collected from a mix of different locations from Greece and Wales and showed a surprising variance in species, where the top 15 most active samples were from 11 different species (Table 4-10).

Table 4-10 A summary of the top 15 most active sponge extracts against MSSA determined using disc diffusion assay ($p < 0.05$). Unstudied species targeted highlighted in bold.

Sponge ID	Species	Location
As1	***Chondrosia reniformis***	Greece
As6	***Chondrilla nucula***	Greece
As13	*Aplysina aerophoba*	Greece
Af3, Af6, Af14	*Agelas oroides*	Greece
Af11	***Ircinia variabilis***	Greece
Af13	***Hemimycale columella***	Greece
Af17	***Petrosia ficiformis***	Greece
2w6, 2w8	*Hymeniacidon perleve*	Wales, UK
2w9, 2jo2	***Amphilectus fucorum***	Wales, UK
2w15	*Halichondria panicea*	Wales, UK
2w20	***Dysidea fragilis***	Wales, UK

The species *Agelas oroides* was the most prolific producer of active extracts where all but one of the extracts featured in top 15 most active extracts (Table 4-10). The remaining extract placed 16th in order of activity. This was expected as the *Agelas* species and particularly *Agelas oroides* are known producers of antibacterial compounds (Forenza *et al*. 1971; Eder *et al*. 1999; Xiong Fu *et al*. 1998). Another eight of the targeted species investigated also showed promising antibacterial activity, and were therefore good leads for further investigation, increasing the chance of isolating a novel compound. In summary, 14

of the 25 (56%) Welsh samples and 17 of the 40 (43%) Greek extracts tested displayed activity, when compared to literature these figures suggest the sponge species targeted are particularly active with less than 5% of some Alaskan sponge samples (Abbas *et al*. 2011) and 20% of some Moroccan sponge samples (Rifai *et al*. 2005) displaying activity against MSSA.

4.4.6.3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Welsh and Greek marine sponge extracts against MRSA and E. coli

MIC and MBC were determined for all acetone and methanol extracts against MRSA and *E. coli*. Only one hexane extract was tested due to its observed activity in the previous tests (sections 4.4.6.1 and 4.4.6.2). Further hexane extracts were not tested due to the uneven nature of any suspension formed with water or DMSO. The MIC and MBC of extracts that were active against MRSA and *E. coli* are shown in Figure 4-19 and Figure 4-20, respectively.

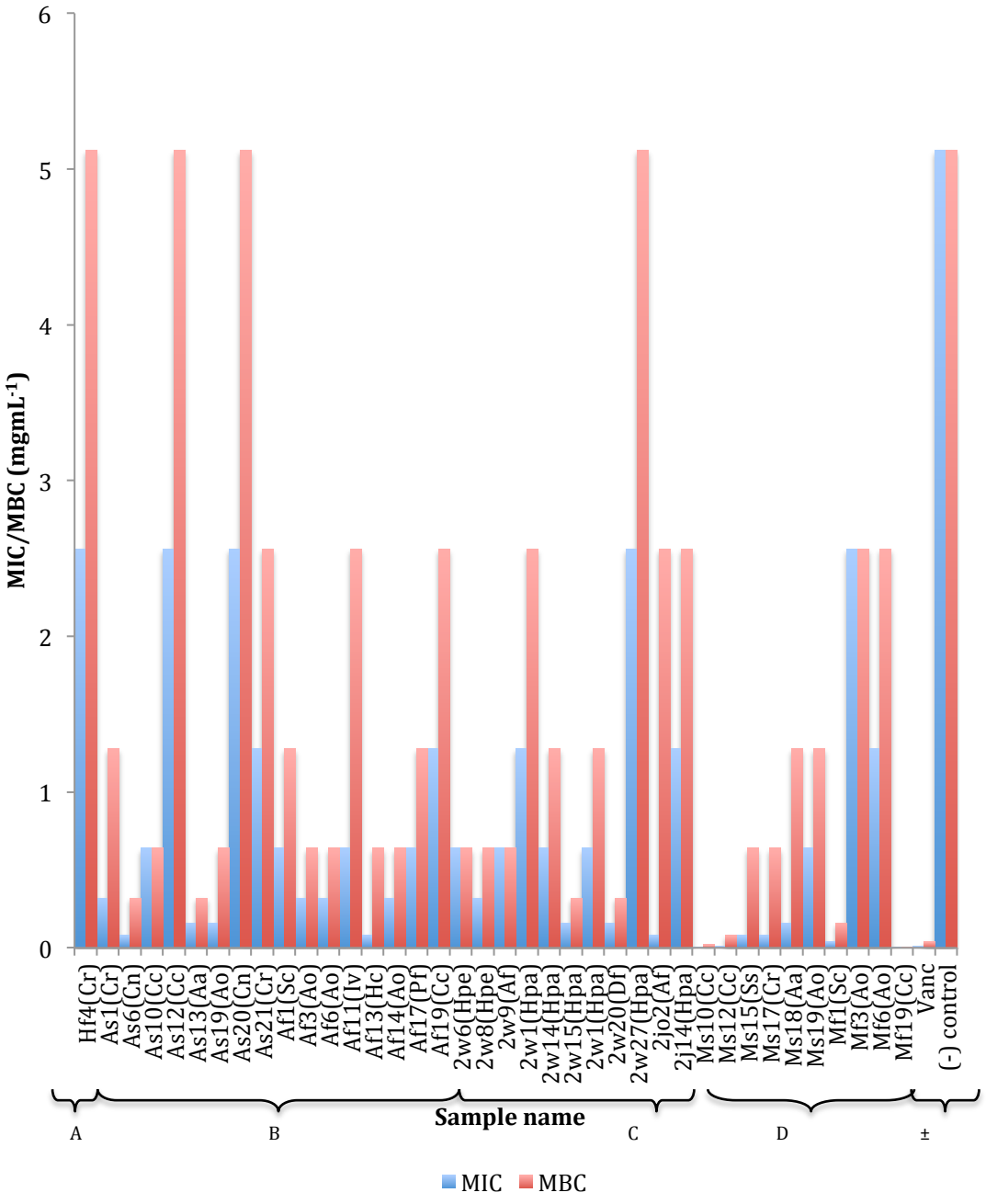


Figure 4-19 MIC and MBC of Greek and Welsh sponge extracts against MRSA using microdilution method. Each result was completed in triplicate. A = Hexane extract of Greek species. B = Acetone extracts of Greek species. C = Acetone extracts of Welsh species. D = Methanol extracts of Greek Species. (±) = Positive (Vancomycin) and negative control. Species abbreviations: Hpe = *H. perleve*, Hpa = *H. panicea*, Af = *A. fucorum*, Df = *D. fragilis,* Cr = *C. reniformis,* Ss = *Sarcotragus sp*, Cn = *C. nucula*, Cc = *C. Crambe*, Aa = *A. aerophoba*, Pf = P. *ficiformis*, Sc = *S. cunctatrix*, Hc = *H. columella,* Ao = *A. oroides* and Iv = *I. variabilis*. No activity observed is indicated by a value = 5.12 mg mL^{-1} (Double concentration of first well).

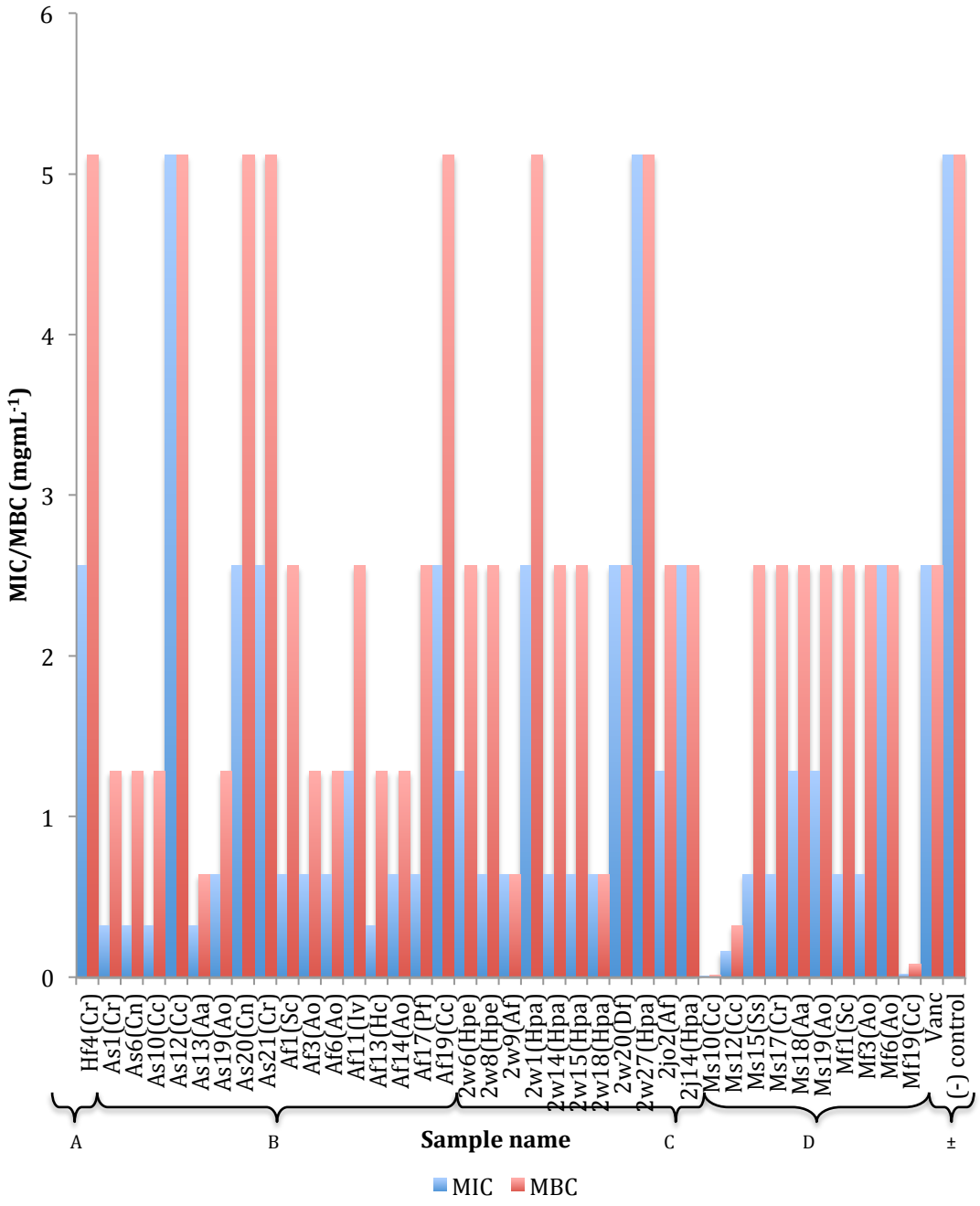


Figure 4-20 MIC and MBC of Greek and Welsh extracts against *E. coli* using microdilution method. Each result was completed in triplicate. A = Hexane extract of Greek species. B= Acetone extracts of Greek species. C = Acetone extracts of Welsh species. D = Methanol extracts of Greek Species. (±) = Positive (Vancomycin) and negative controls. Species abbreviations: Hpe = *H. perleve*, Hpa = *H. panicea*, Af = *A. fucorum*, Df = *D. fragilis,* Cr = *C. reniformis,* Ss = *Sarcotragus sp*, Cn = *C. nucula*, Cc = *C. Crambe*, Aa = *A. aerophoba*, Pf = P. *ficiformis*, Sc = *S. cunctatrix*, Hc = *H. columella,* Ao = *A. oroides* and Iv = *I. variabilis*. No activity observed is indicated by a value = 5.12 mg mL^{-1} (Double concentration of first well).

The MIC and MBC of extracts against MRSA and *E. coli* showed similar activity profiles (Figure 4-19 and Figure 4-20) with slightly less antimicrobial activity recorded against the gram-negative bacterium *E. coli*, which corresponded with results from the bioautographic assay (section 4.4.6.1). Any results recorded as 5.12 mg mL^{-1} showed no activity in the tests completed this score was recorded, as the concentration is higher than that of the first well (2.56 mg mL^{-1}) and it showed the most meaningful results when displayed in a graph. Both the Welsh extracts and Greek extracts showed good antimicrobial activity against both bacteria.

Overall, 11 of 50 (22%) of Welsh extracts and 27 of 79 (34%) of Greek extracts displayed activity against MRSA; whilst 10 of 50 Welsh extracts (20%) and 26 of 79 Greek extracts (33%) showed slightly less activity against *E. coli*. As no other research project looking at marine sponges has sequentially extracted directly from sponge material (giving three times the number of crude extracts), the fairest comparison to literature would be to look to see if any extracts from a collected sponge species showed activity. From 25 Welsh samples tested 11 different collected samples displayed activity against MRSA (44 %) and 10 against *E. coli* (40 %). From the 39 different Greek samples tested 20 different collected samples displayed activity against MRSA (51 %) and 19 against *E. coli* (48 %). Both of these figures are more than comparable to the activity identified in a study of Moroccan sponges which displayed 20% and 40% against MSSA and *E. coli* respectively (Rifai *et al*. 2005). This however is not a perfect comparison as different strains of bacteria were used.

No antimicrobial activity was observed in any of the methanol extracts from the Welsh samples even though activity was observed in the bioautographic assay. This may be due to the increased sensitivity of bioautographic technique in comparison to the MIC assay due to the increased purity of compounds. The concentration of the active products may not have been high enough to display activity in a MIC well assay but high enough when separated on a TLC plate and tested directly on a bacterium using the bioautographic overlay method (Begue and Kline 1972). It is also worth noting that in the bioautographic assay Welsh methanol extracts showed no activity towards *E. coli* but only to MSSA and when the microdilution assay was completed a different strain of bacterium was used, in the form of drug resistant MRSA. Therefore, MRSA may have been resistant against some of the active components in the methanol extracts of sponge samples from Wales. Some strong activity was however observed with Greek methanol extracts, with extracts of *Crambe crambe* Ms10, Ms12 and Mf19 displaying the highest antimicrobial activity against both bacterial

strains. These extracts displayed antimicrobial activity that was equivalent or better than Vancomycin, one of the standard hospital treatments for MRSA (Hope *et al*. 2013) and when extracts were grouped by species the methanol extracts of *Crambe crambe* showed significantly the best activity ($p < 0.05$) of any extracts.

Although the active Greek extracts seemingly showed higher activity than the Welsh extracts (Figure 4-21), this was not statistically significant ($p > 0.05$). This means the activity observed from sponge species from Wales was similar to those found from the warmer waters of Greece, which may not have been expected due to the increase of diversity in warmer waters (Ausubel *et al*. 2010).

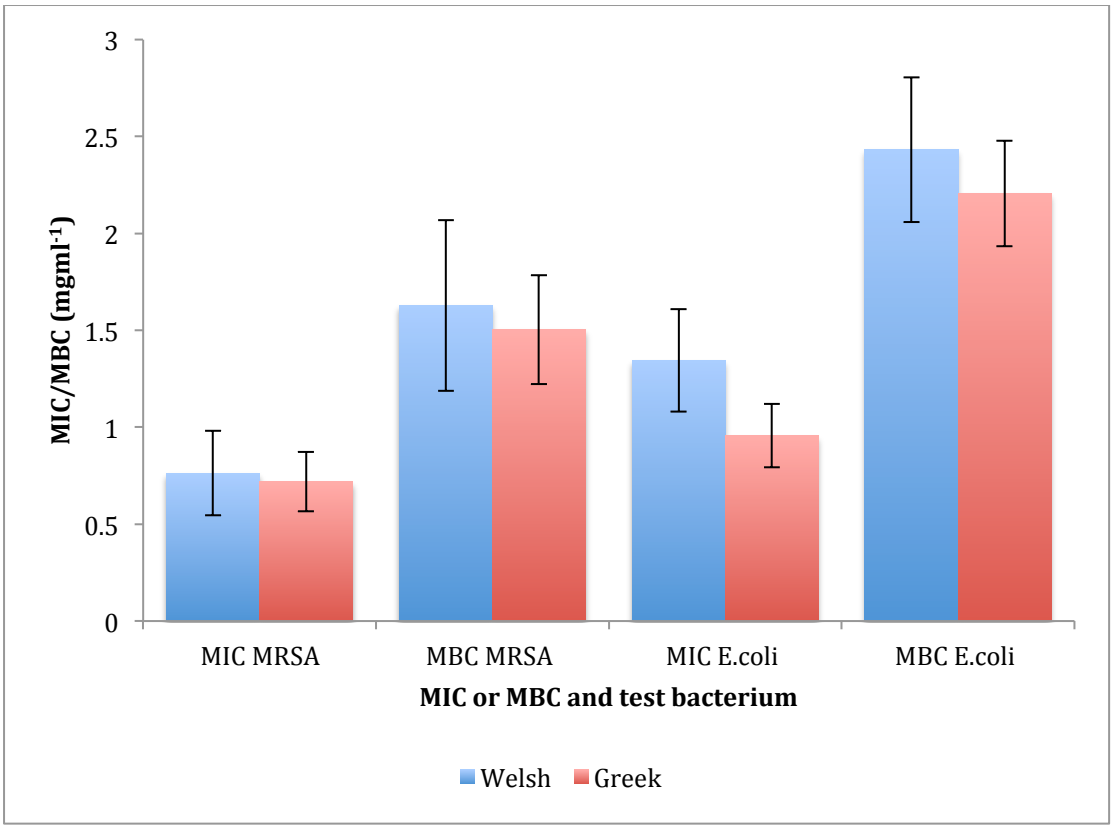


Figure 4-21 Comparison of the activity of the average active Greek and Welsh extract. Welsh active samples MRSA (n = 11), Welsh active samples *E. coli* (n = 10), Greek active samples MRSA (n = 27) and Greek active sample *E. coli* (n = 26), error bars = standard error of mean.

4.5 Conclusion

Several chemically uncharacterised species of sponge have been identified and collected along with some chemically well-characterised species for a direct comparison of antimicrobial activity. Samples were collected from both Greece (warm temperate water) and Wales (cold temperate water) and were extracted using the sequential extraction method and their extract profiles were compared. A significant difference was observed between the profiles of extracts from Greece and Wales with a larger proportion of yield from Welsh sponges extracted in methanol. This average extraction profile information can be used to direct future sponge sample collection to allow prediction of the quantity of starting material required for further activity testing and compound identification.

The extracts were then tested against clinically relevant bacteria MSSA, MRSA and *E. coli* for activity using different assays. The bioautographic technique identified antimicrobial activity against MSSA and *E. coli* in a large proportion of extracts studied. This data also provided information on the polarity of active chemicals within an extract and the variance in this result suggests that the activity within samples could be attributed to different chemical entities. Preliminary information from this study was used to direct experiments reported in subsequent chapters, which employed various separation techniques in order to isolate and identify active compounds within an extract.

Quantifiable antimicrobial activity measurements were also performed on the sponge extracts with good activity observed against MRSA and *E. coli*. In some cases, the extracts were as effective or more effective than Vancomycin, the standard treatment for MRSA. This is very encouraging for the efficacy of the compounds present as the extracts are not pure compounds and still represent a crude mix. The results of the quantifiable assays also showed direct correlation to the bioautographic results, when the same bacterial species was used, thus validating the use of the bioautographic method as a pre screen.

Chapter 5
Activity guided isolation and characterisation of bioactive components from Welsh and Greek sponges

5 Activity guided isolation and characterisation of bioactive components from Welsh and Greek sponges

5.1 Chapter introduction

5.1.1 Collected Welsh sponges chemical background

A search was completed on MarinLit (Mar 2015) to identify any 'new' compounds isolated from the sponge species collected from Wales (Table 5-1).

Table 5-1 Summary of the number of compounds identified when limiting by taxonomy on MarinLit by sponges species collected.

Species name	N° of compounds identified in MarinLit
Halichondria panicea	43
Suberites ficus	0
Amphilectus fucorum	0
Hemimycale columella	0
Tethya citrina	0
Dysidea fragilis	70
Hymeniacidon perleve	12

A surprising variation was observed in the number of compounds identified from each sponge species with no compounds at all being previously isolated from some collected species (*Amphilectus fucorum, Hemimycale columella Suberites ficus* and *Tethya citrina*). All of the compounds found on MarinLit matching the parent sponge *Hymeniacidon perleve* were all actually extracted from associated fungi or bacteria (Song *et al*. 2014; Zhao *et al*. 2011; X. Wang *et al*. 2014). However limiting by genus you would expect to find brominated alkaloids such as hymenidin, and hymenin (section 1.4.3), sterols or the proline rich heptapeptides such as hymenamide (section 1.4.1).

MarinLit identified 43 compounds from *Halichondria panicea* however similar to *Hymeniacidon perleve* a significant number of compounds where actually isolated from bacteria or fungi,(Mitova *et al*. 2008; Schneemann *et al*. 2010) this was previously noted when completing the general literature search (section 4.4.2). However some compounds have been extracted directly from the sponge including the paniceins and halipanicine (Cimino *et al*. 1973; Nakamura *et al*. 1991)(Figure 5-1).

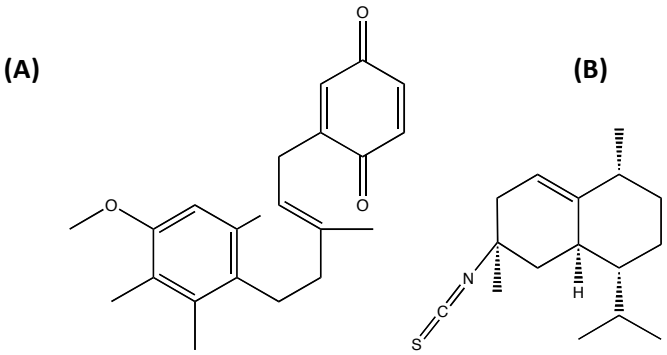


Figure 5-1 Structure of (A), Panicein A and (B) Halipanicine.

MarinLit identified 70 compounds from *Dysidea fragilis*, making it the most thoroughly chemically characterised of all the studied Welsh sponges. Some interesting compounds identified include the diketopiperazines the dysamides, the sesquiterpene spirofragilin and the cytotoxic dysidaminones (Su *et al*. 1993; Yu *et al*. 2006; Jiao *et al*. 2014) (Figure 5-2).

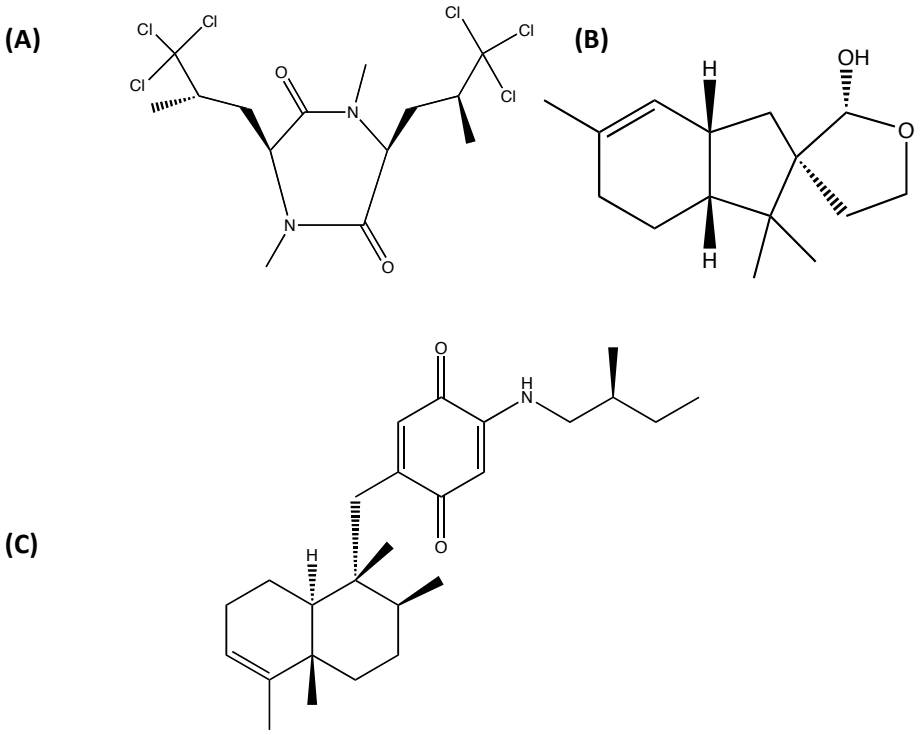


Figure 5-2 Structure of (A) Dysamide A; (B), spirofragilin and (C), Dysidaminone B.

5.1.2 Dereplication and structure elucidation

The overall aim of the research presented in this thesis was to screen marine sponges for novel drug-like molecules. Two fundamentally important parts of this process were dereplication and structure elucidation. Antibacterial activity was confirmed in chapter 4 using a variety of approaches and the bioautographic overlay assay indicated that multiple compounds within the natural product extracts contributed to the activity observed.

It is well established that marine sponges contain a complex mixture of natural products and numerous antibacterial compounds have been previously isolated from sponges (Table 5-2). It is therefore vitally important to eliminate previously identified molecules as potential leads as early as possible in the drug discovery process as it can take up to two years to structurally identify a complex molecule such as marinomycin (Kwon *et al*. 2006). This process is commonly referred to as dereplication (Lee *et al*. 2001) and aims to prevent rediscovery and inappropriate use of time and resources.

Table 5-2 Selected antibacterial compounds and their original source sponge.

Sponge	Bioactive metabolite	Activity type	Reference
Acanthella sp.	Kalihinol-A	Antibiotic	(Chang *et al*. 1984)
Agelas dispar	Aminozooanemonin	Antibacterial	(Cafieri, Fattorusso and Taglialatela-Scafati 1998a)
Agelas dispar	Pyridinebetaine A	Antibacterial	(Cafieri, Fattorusso and Taglialatela-Scafati 1998a)
Agelas mauritiana	Sceptrin	Antimicrobial	(Bernan *et al*. 1993)
Agelas nakamurai	Ageliferine	Antibacterial	(Eder *et al*. 1999)
Agelas nakamurai	Debromosceptrin	Antibacterial	(Eder *et al*. 1999)
Agelas nakamurai	Nakamuric acid	Antibacterial	(Eder *et al*. 1999)
Agelas sp.	Agelasine I	Antimicrobial	(Xiong Fu *et al*. 1998)
Discodermia kiiensis	Discodermin A	Antimicrobial	(Matsunaga *et al*. 1985)
Ianthella basta	Bastadin	Antimicrobial	(Pettit *et al*. 1995)
Jaspis sp.	Psammaplin	Antibacterial	(D. Kim et al. 1999)
Jaspis wondoensis	Wondosterols	Antimicrobial	(Ryu *et al*. 1999)
Poecillastra wondoensis	Wondosterols	Antimicrobial	(Ryu *et al*. 1999)
Psammaplysilla purpurea	Bastadin	Antimicrobial	(Carney *et al*. 1993)
Subera creba	Aeroplysinin I	Antibacterial	(Debitus *et al*. 1998)
Subera creba	Dibromoverongiaquinol	Antibacterial	(Debitus *et al*. 1998)
Verongia aerophoba	Aeroplysinin I	Antibacterial	(Fattorusso *et al*. 1970)

There are multiple approaches to achieve dereplication. Firstly, a technique to effectively separate the compounds from within a complex mixture is required, most commonly through a chromatographic method such as HPLC, TLC or HPCCC. The next stage is to gain physicochemical data for the compounds separated, such as their mass or information about their structure. Spectroscopic techniques are routinely used (*e.g*. MS and NMR respectively)

and can be coupled directly with the separation technique *e.g.* HPLC-MS (Colorado-Rios *et al*. 2013) or HPLC-NMR (Dias and Urban 2009). These 'hyphenated' techniques give rapid and accurate results that can be completed in most laboratories and also directly compared to structure databases as the primary step of dereplication.

The starting material collected was extremely limited for some species (less than 5 g), which led to development of methods chosen. This project aimed to combine various separation techniques to show how useful information can be gained from marine sponges using limited starting materials.

5.1.3 Thin layer chromatography coupled with mass spectrometry (TLC-MS) as a dereplication strategy

Thin layer chromatography (TLC) is a simple, efficient and economic method of separating natural products that is often used for direct analysis of crude samples that have undergone little or no purification (Cheng *et al*. 2011). The bioautographic overlay technique allows direct visualisation of activity on an eluted TLC plate (Hamburger and Cordell 1987). A metabolic stain layered onto a TLC plate can identify any areas where no bacteria have grown, confirming antibacterial activity of the separated compound (Begue and Kline 1972), allowing a direct comparison between Rf and biological activity (Kasote *et al.* 2015).

Historically, indirect methods were used to identify or remove separated compounds from a TLC plate and samples had to be extracted and then re-concentrated (I. D. Wilson 1999; Cheng *et al*. 2011). This is a time consuming method and results in a significant loss of material before dereplication is completed (section 3.4). Rapid and contaminant free direct methods of TLC-MS are now available. Such methods allow instant collection of mass data from identified areas on a TLC plate (Figure 5-3).

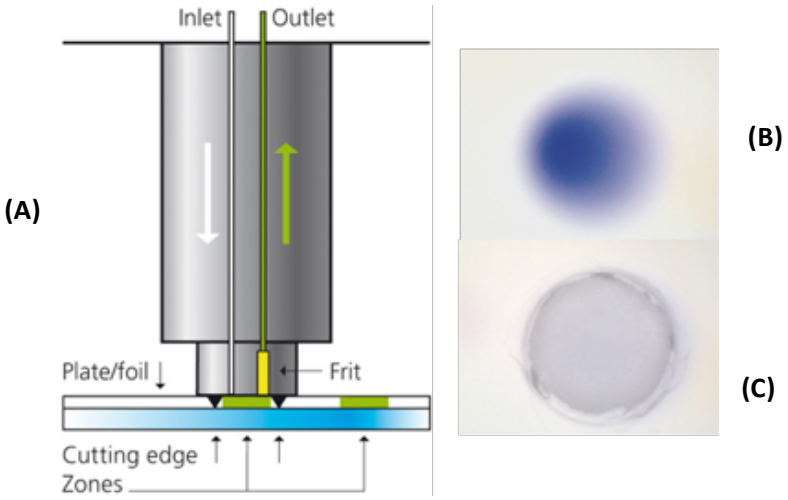


Figure 5-3 An illustration of TLC-MS instrumental analysis. (A) Scheme of the extraction piston. (B) Area of interest before and (C) following extraction (Taken from CAMAG, Omicron research Ltd, UK).

When used in tandem with bioautographic TLC, this technique allows rapid dereplication of known compounds directly, following the identification of areas of activity on a TLC plate (Kasote *et al*. 2015). A key advantage of this technique over the more commonly employed LC-MS method of separation is that non-UV active compounds will also be identified. While the mass of any product (including its fragmentation and/or any isotopic patterns) may be able to prevent rediscovery, the disadvantage of this method is that without fragmenting the product through electron-impact MS or applying other spectroscopic techniques, such an NMR, definitive identification may not be possible (Yang *et al*. 2013). This technique allows the dereplication of antibacterial compounds straight from a TLC plate and uses very little material so is therefore perfect for a pre-screen as identified in section 3.4.4.

5.1.3.1 Data mining using marine specific databases

The success of any approach to dereplication relies heavily on the quality of the chemical library used (Hughes and Fenical 2010) and this project has predominantly used the marine natural product specific library *MarinLit*. Many natural product databases are now commercially available but it has been suggested that using marine specific natural product database aids the acceleration of identification of isolated compounds (Gassner *et al*. 2007). Examples of additional marine specific sources include *The Chapman and Halls Dictionary of Marine Natural Products* (DMNP); which contains over 35,000 compounds of marine origin (Blunt and Munro 2014); NAPRALERT^{TM} a comprehensive database that records all natural products and *AntiBase* (the Natural Compound Identifier); which contains over 40,000 compounds of natural origin.

In 2014 the Royal Society of Chemistry (RSC) re-launched *MarinLit*, as an Internet based resource. This database was originally set up as an in-house database for marine natural products at the University of Canterbury Marine Group in the 1970s. *MarinLit* has now been directly linked to RSC's existing database ChemSpider and covers over 24,000 marine compounds from 26,000 journal articles and was used by the project as its primary database for dereplication.

In this research study, it was expected that some known compounds would be rediscovered. However, it may be possible that these could still be good chemical leads if they exhibited the desired antibacterial activity and if they have not been previously explored for this therapeutic indication and are compliant with Lipinski's rules (section 3.1.3).

5.1.4 Molecular networking as a dereplication strategy

Dereplicating early in the drug discovery process minimises time, effort and expense. As discussed earlier, most strategies for dereplication involve hyphenated techniques such as HPLC-MS, HPLC-NMR and HPLC-MS-NMR. These methods utilise the fact that structurally similar or identical molecules share physicochemical characteristics. However, even when multiple characteristics are verified, full elucidation often occurs much later in the workflow or may prove impossible to determine. MS dereplication is an essential part of natural product dereplication workflow and the characterisation and identification of known molecules. It is a more sensitive technique than NMR but parent mass alone does not provide enough refinement of results when searching databases such as *MarinLit* or *SciFinder* (section 3.4.2.6). Tandem mass spectrometry (MS/MS) breaks down both the parent molecule and its fragments into additional fragments. The resulting fragmentation pattern or fingerprint can then be compared to similar compounds with the assumption that searching for similar patterns allows more resolution in dereplication than the parent mass alone (Yang *et al*. 2013). MS/MS is a method of dereplication that is increasingly used, particularly in marine natural products with the introduction of the Global Natural Products Marine Social Networking (GNPS) (GNPS 2015) .

This project aimed to use the fragmentation data from MS/MS to fingerprint each extract and isolate and compare them directly to one another and some known marine natural products. The resulting *molecular network* will not only work as a method of dereplication but also as a method of finding new leads for novel compounds by partially identifying species similar to known compounds. This would make full structural characterisation easier

and also identify potential novel leads that show no similarities to known compounds. By visualising this information in the form of a map-like diagram, the tedious and time-consuming task of directly comparing spectra and looking for similarities is avoided. This technique could be extremely powerful when combined with TLC-MS/bioautography to gain more structural information about the active compounds again using very limited material.

5.1.5 High performance counter current chromatography (HPCCC) as an alternative method of separating active compounds from marine sponges

Logistical and ecological reasons limited the quantity of sponge material collected. Hence, it was essential that every gram of material collected should be utilised efficiently to yield the best results. When analysing the constituents of marine sponges, many active metabolites are found in concentrations of less than 0.00001 % (Radjasa et al. 2007). Therefore any techniques used for separation, identification and dereplication need to be extremely sensitive. The lack of sufficient material emphasises one of the limiting factors of traditional separation techniques such as LC and HPLC, where material is lost by irreversible binding to the stationary phase.

High performance counter current chromatography (HPCCC) is an alterative approach to HPLC and is unique due to its ability to isolate high yields and volume of pure compounds from crude natural product extracts (Martin and Synge 1941). HPCCC is uncommon but over the past 15 years, has become an increasingly popular separation technique for drug discovery; mostly due to improvements in the hardware technology (Sutherland and Fisher 2009). It is still a relatively unexploited technique for characterisation of marine sponges with only a few studies reporting use of this separation technique (Jadulco 2002). Similar to HPLC, HPCCC separates compounds based on their polarity but uses immiscible solvent system pairs, of differing polarity, to produce a biphasic interface at which compounds are separated by their solubility between the two solvents systems (Sutherland and Fisher 2009). HPCCC requires the solvent interface to be maintained over as large an area as possible. This is engineered within the system by wrapping PTFE tubing, through which the sample flows, around a bobbin, which is rotated at high speed. HPCCC is ideal for samples where there is a low quantity of starting material as little or no material is lost following separation. Unlike HPLC, HPCCC is scalable allowing the separation to be refined with a small quantity of material and then scaled up with any further material (DeAmicis *et al*. 2011).

When used to its potential, HPCCC is an extremely powerful tool for the separation of crude extracts. Limiting factors are the machine hardware and the difficulties in selecting a suitable solvent system as compounds cannot be restricted to one phase (DeAmicis *et al*. 2011).

In this chapter, research was conducted to find an effective solvent system for initial separation of all marine sponge extracts obtained from the Greek and Welsh collections. Two different chromatographic techniques were linked; the source of the antibacterial activity was visualized on a TLC plate using the bioautographic overlay assay and then various solvent combinations along the HEMWat (Hexane, Ethanol, Methanol and Water) scale were evaluated to determine the optimum solvent system for HPCCC.

5.1.6 The potential effect of environmental competition from differing sponge species on the production of secondary metabolites

Aplysina aerophoba is one of the most thoroughly studied sponges with research reporting the effect temporal variation has on the secondary metabolite production (Sacristán-Soriano *et al*. 2012); secondary metabolite variation within sponge material (Sacristán-Soriano *et al*. 2011) and the effect of disease on chemical pattern (Webster *et al*. 2008). A thorough literature review revealed no published studies on secondary metabolite variation between two specimens of *A. aerophoba* harvested from competitive and non-competitive environments. As this species was abundant at the Samos and Fourni collection sites, it was easily found in these variable conditions. Therefore, this sponge species was chosen as the prime candidate for research on the effect of environmental competition on the production of secondary metabolites. While this species has been thoroughly studied in other aspects, further research into the environmental factors that regulate chemical variation could aid in the discovery and supply of novel bioactive compounds. The main reason for investigating this difference was to eventually streamline the collection of sponges to those with the highest yield of secondary metabolites by targeting sponges in specific spatial environments.

5.2 Chapter aim and objectives

The primary aim of this chapter was to screen marine sponges for novel antibacterial leads. The objectives of this chapter were to:

1. Describe the combination of TLC-MS and bioautographic TLC as methods of dereplication against a marine specific natural products database and identifying novel antibacterial leads using little starting material.
2. Generate a molecular network, allowing direct dereplication against standards, using HPLC-MS/MS from extracts demonstrating antibacterial activity.
3. Purify selected biologically active extracts using an optimised HPCCC solvent system.
4. Dereplicate and identify antibacterial leads by comparing molecular network matches to compounds isolated from HPCCC – bioautographic TLC-MS.
5. Assess the effect of environmental competition on the production of secondary metabolites.

5.3 Materials and methods

5.3.1 Bacterial cultures

Cultures used for antibacterial testing were MSSA (NCIMB 9518), *E. coli* (NCIMB 12210) and MRSA (NCTC 11939), which were obtained from Cardiff University's culture collection. All manipulations were completed in a laminar flow cabinet. All equipment used for bacterial analysis were either purchased sterile or sterilised by autoclaving at 121 °C for 15 min at 1bar.

5.3.2 Analytical thin layer chromatography (TLC)

Analytical thin layer chromatography was performed using TLC silica gel F_{254} by Merck (Darmsradt, Germany). The solvent systems used were 8:2 (hexane : acetone) for hexane extracts, 6:4 (hexane : acetone) for acetone extracts and 8:2 (dichloromethane : methanol) for methanol extracts, unless stated otherwise. Each plate was eluted at room temperature using a glass chamber and 50 mL of the solvent system. Eluted plates were then visualised using a UV lamp (UVGL-55 254/365 nm, UVP, US) at wavelengths of 254 nm and 365 nm. A picture was taken of the plate in daylight and under UV fluorescence for comparison with the bioautographic overlay assay results.

5.3.3 Direct mass spectrometry from a thin layer chromatography plate (TLC-MS)

5.3.3.1 Bioautographic technique prior to TLC-MS

The bioautographic overlay method (section 4.3.2) was repeated on larger plates against MRSA (NCTC 11939) with vancomycin as a control. This allowed improved visualisation of activity with products separated to a greater resolution due to the increased distance of separation. The completed plates, with visual zones of inhibition, were then directly compared to a newly separated plate. Any areas of interest were marked and numbered on the newly separated plate prior to TLC-MS. The intensity of activity was also recorded as a guide of activity even though this is not quantifiable.

5.3.3.2 TLC-MS

Direct TLC-MS was completed using the TLC-MS interface (CAMAG, Switzerland) in-line with a MicroTOF electrospray mass spectrometer (Bruker, Germany) in positive mode giving low-

resolution baseline peaks. The monoisotopic mass of the 'active compound' was then estimated based on the possible ionisations of the observed peak ($(M + H)^+$, $(M + Na)^+$ or $(2M + Na)^+$).

5.3.4 Data mining

5.3.4.1 Identification of novel compounds using database analysis

The masses for the expected molecular ions from the TLC-MS were used to search both *MarinLit* and *Scifinder* databases. *SciFinder* is a comprehensive but non-specialised database; direct comparison between the two databases was used to assess *MarinLit's* usefulness in data mining marine specific natural products. The variation for molecular mass was set at ± 0.1 to narrow down the number of matches. To obtain the most useful results, refinements had to be put in place for each molecular weight searched in *SciFinder*, with the first refinement by 'natural products' and the second refinement by 'marine'. If no matches were identified with the mentioned stipulations, the drug was considered a novel compound (Figure 5-4).

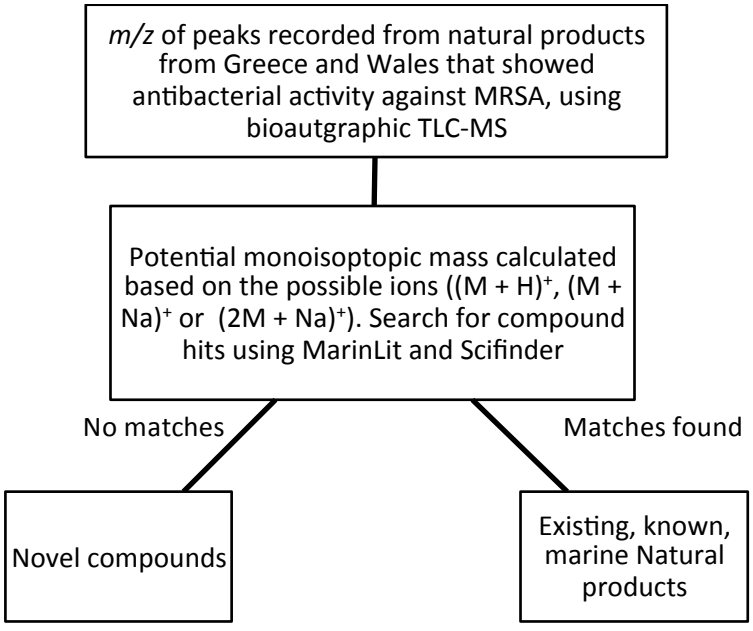


Figure 5-4 A schematic flow diagram of data mining steps employed post identification of active parent mass using bioautographic TLC-MS. Activity observed against MRSA.

5.3.4.2 Known compound lead refinement

When compound mass alone is searched in any database, a large number of matches were displayed. This large number of matches was then refined by parameters and the following series of refinement procedures were followed when searching *MarinLit*:

- **Taxonomy**: *MarinLit* allows taxonomic refinement in any level of taxonomic classification from domain to species. Results where then refined by genus and if no matches were found post refinement, the extract was then categorised as a 'possible novel compound'.
- **Mass data:** Mass spectrometry data not only provided a searchable parent mass but also interpretation of the appearance of neighbouring peaks helped the identification of potential constituents. Halometabolites are common in marine sponges and their presence can be confirmed with the visualisation of isotopic peak patterns. If a halogen is present the parent peak is split into isotopic peaks allowing visual identification of the number and type of halogen present. For example, in a mass spectrum of a single brominated compound, the appearance of two peaks would be expected with a 1 : 1 intensity ratio, with peaks two mass units apart (M : M + 2). If two bromine functional groups are present, an intensity ratio of 1:2:1 would be expected, with peaks two mass units apart (M : M+2 : M+4 respectively). M in both cases represents the monoisotopic peak (Figure 5-5). Such information can then be added to the refinement criteria in *MarinLit* by insertion as 'known data' *i.e.* the number of bromines/chlorines in the molecular formula box.

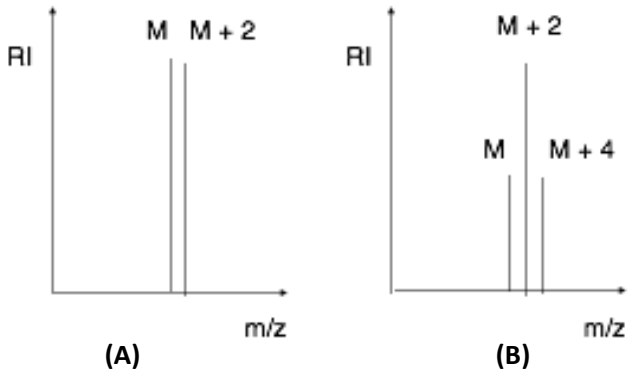


Figure 5-5 Example mass spectra of halogenated split peaks: (A) Single bromine and (B) Two bromines. M represents the monoisotopic mass.

- **Lipinski's Rule of Five** is the general rule of thumb, which describes the optimum physicochemical properties for oral bioavailability (section 3.1.3). As such, it is often

used a proxy to define molecules with good potential for drug development (Lipinski *et al*. 1997). This study limited compounds for further study to those with MW of less than 550. There was also a *ChemSpider* link within *MarinLit*, which allowed limitations of physiochemical properties, and this was also utilised as a method of refinement using other Lipinski criteria.

5.3.5 High performance counter current chromatography

5.3.5.1 HPCCC method development

The sample solution was prepared by dissolving the extract in the appropriate phase to 100 mg mL^{-1}. A volume of 3mL of sample was injected into a loop of 3.71 mL and injected into the coil spinning at 1600RPM at 30 °C. Two columns were used, to increase surface area of contact, with a flow rate of 4 mL min^{-1}. Approximately 20 mL of stationary phase was extruded during equilibration and flow was run for 80 min before extrusion with the starting stationary phase. Samples were taken every minute during the primary run and the column was then extruded at 40 mL min^{-1}, taking a sample every 6 s. UV detection was recorded at 210 nm, 230 nm and 254 nm.

5.3.5.2 Antibacterial activity based solvent system selection

HPCCC solvent systems were determined by utilising the data from the bioautographic technique. Samples were suspended at 10 mg mL^{-1} in different ratio of two-phase solvent systems along the HEMWat scale, starting with the solvents the extracts were isolated with. The presence of active compounds in both phases was confirmed using TLC. A good solvent system was considered to be one where the active compounds identified were equally distributed between the two phases (Figure 5-6).

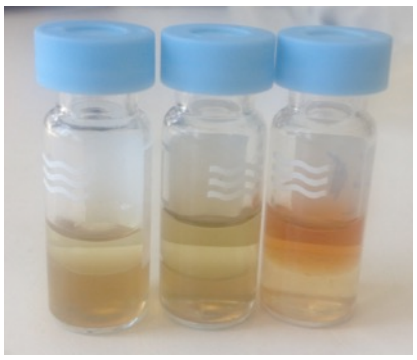


Figure 5-6 Digital image of marine sponge extracts suspended in biphasic systems along HEMWat scale. A visual difference was readily observed in the phase location of antibacterial products, which was confirmed by TLC.

5.3.5.3 Preparation of marine sponge extracts prior to HPCCC

The sample solution was prepared by dissolving the extract in the appropriate phase to a concentration of 50 to 100 mg mL^{-1}.

5.3.5.4 HPCCC template run

3mL of each sample was injected into a loop of 3.71 mL and samples were separated by injection into the coil spinning at 1600 rpm at 30 °C. Two columns were used, to increase surface area of contact, with a flow rate of 4 mL min^{-1}. 20-30 mL of stationary phase was extruded during equilibration and flow was allowed to run at different times dependent on the extract being separated. Fractions were continuously collected every minute during the primary run and the column was then extruded at 40 mL min^{-1} using the starting stationary phase, where fractions were collected every 6 s. Detection was recorded at a UV absorbance of 254 nm and 365 nm.

All fractions were then reduced by half using a miVAC duo centrifugal concentrator (Genevac, USA) and tested for activity using the bioautographic technique. Neighbouring fractions with activity at the same Rf value were combined and dried to a solid form using the centrifugal concentrator. Identification of the active compounds within these fractions was completed using TLC-MS and HPLC-MS/MS.

5.3.5.5 Assessing the antibacterial activity of an extract using the minimal inhibitory concentration (MIC) microtitre broth dilution method

The activity of the fractions, separated by HPCCC, was compared to that of the crude extracts by testing their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the method described in section 4.3.4.

5.3.6 HPLC – MS/MS and molecular networking

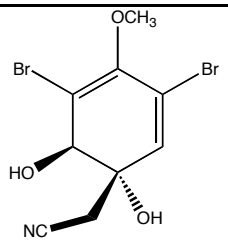
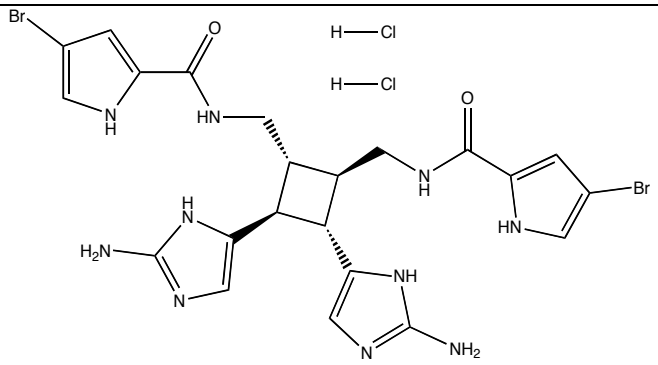
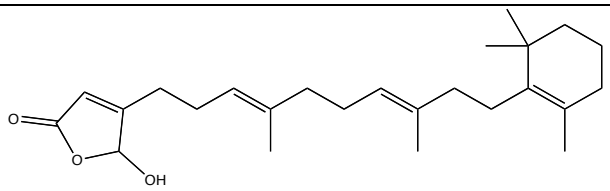
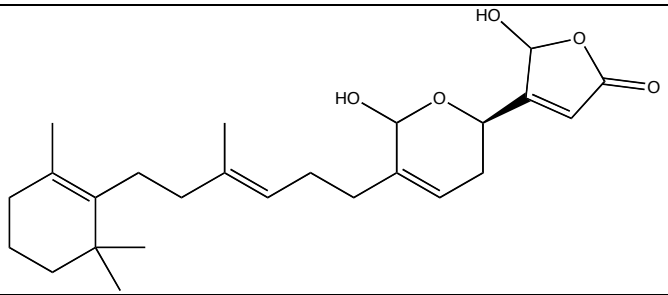
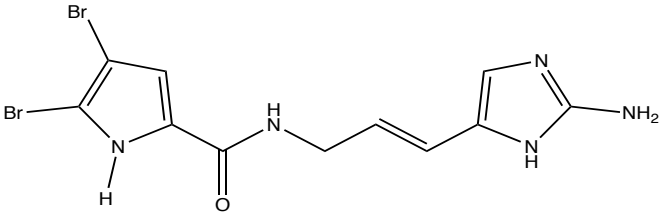
5.3.6.1 Sample preparation and standards

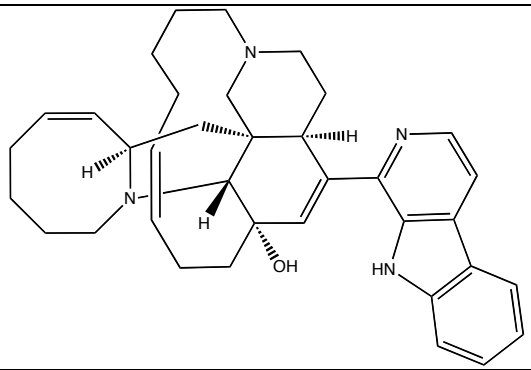
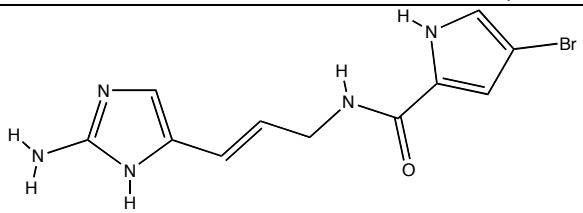
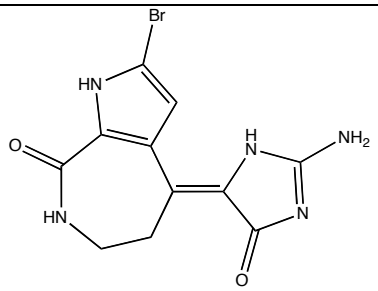
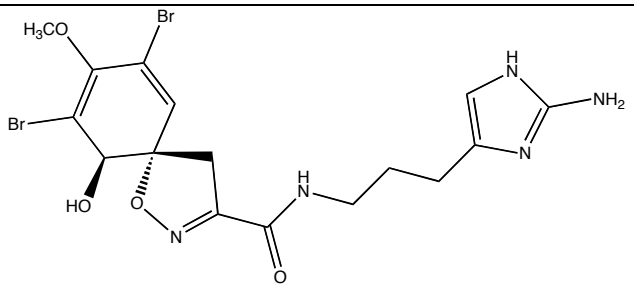
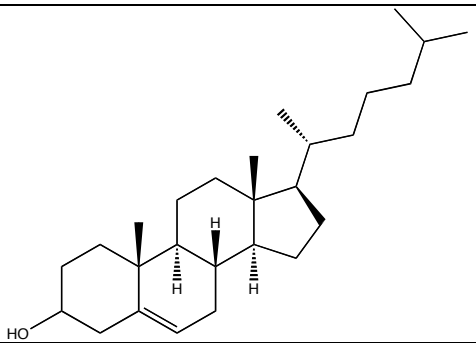
Samples were diluted in the appropriate solvent (HPLC grade methanol, acetonitrile or water) to a concentration of 50 μg mL^{-1}.

5.3.6.2 Selection of standards

Standards were selected based on their frequency of isolation from sponges, with a diverse selection of groups of compounds including imidazoles, sesterterpenoids, guanidine alkaloids and various other brominated alkaloids. Compounds with molecular weights generally less than 550 were chosen (Table 5-3). Sceptrin was the largest molecule selected at MW 620. Larger molecules, *e.g.* peptides, were disregarded as discussed in section 3.1.3.

Table 5-3 Sponge natural products chosen as standards for a molecular network. Monoisotopic masses are reported.

Name of standard, supplier and mass	Structure of compound	'Type' of compound, activity and isolation information
(+)-Aeroplysinin-1 Enzo Life Sciences, UK Isolated from *Aplysina aerophoba*. MW 336.894897		Antibacterial brominated tyrosine metabolite originally isolated from *Aplysina aerophoba* (Fattorusso *et al.* 1970). Also isolated from other species such as *Subera creba* (Debitus *et al*. 1998).
Sceptrin – dihydrochloride Enzo Life Sciences, UK Isolated from *Agelas Nakamurai*. MW 689.998413 Or 620.06080 without dihydrochloride		Dimeric pyrrole - imidazole alkaloid with antimicrobial activity isolated from *Agelas mauritiana* (Bernan *et al*. 1993).
Luffariellolide Enzo Life Sciences, UK Isolated from *Luffariella sp*. MW 386.2810		Marine sesterterpenoid isolated from *Luffariella sp.*
Manoalide Enzo Life Sciences, UK MW 416.25630		Antibiotic sesterterpenoid originally extracted from the marine sponge *Luffariella variabilis* (de Silva and Scheuer 1980).
Oroidin Enzo Life Sciences, UK Isolated from *Stylissa sp* MW 386.93310		Dibrominated bromopyrrole alkaloid original isolated from *Agelas oroides* (Forenza *et al*.

Name of standard, supplier and mass	Structure of compound	'Type' of compound, activity and isolation information
		1971).
Manzamine A Santa Cruz Biotech, USA MW 548.35150		Antibacterial β-carboline alkaloids originally isolated from a *Haliclona sp.* (Sakai *et al*. 1986).
Hymenidin Santa Cruz Biotech, USA MW 309.022522		Antibacterial monobrominated bromopyrrole alkaloid originally isolated from *Hymeniacidon sp* (Kobayashi *et al*. 1986).
10Z-Hymenialdisine Santa Cruz Biotech, USA. Isolated from sponge *Axinella carteri*. MW 323.00180		Monobrominated guanidine alkaloid isolated from *Hymeniacidon aldis.* (Kitagowa *et al*. 1983).
Aerophobin-2 Santa Cruz Biotech, USA. Derived from *Aplysina aerophoba*. MW 502.98050		Cytotoxic bromotyrosine alkaloid also known to have antibacterial properties isolated from the Caribbean sponge *Aiolochroia crassa.*
Cholesterol Sigma-Aldrich, UK. MW 386.354858		Sterol isolated from *Aplysina aerophoba* in section 3.4.3.2.

5.3.6.3 High performance liquid chromatography coupled with accurate mass spectrometry and parent mass fragmentation (HPLC- MS/MS)

HPLC-MS/MS was completed using UPLC in-line with a QTOF electrospray mass spectrometer (UPLC-ESI-QTOF) (Bruker, Germany).

The LC conditions (on an Ultimate 3000 LC, Dionex, USA) were as follows:

Column: Phenomenex Kinetex 1.7 µm, 50 x 2mm, XB-C18 100A, held at 30 °C. The gradient solvent system is described in Table 5-4.

Table 5-4 LC conditions table describing the gradient of solvents run between the first and fifteenth minute.

Time	% water (A)	% acetonitrile (B)
0 (start)	75%	25%
1	75%	25%
11	5%	95%
14	5%	95%
15 (end)	75%	25%

A 10 µL injection volume of standard for internal calibration (not through column) and sample at 0.5 min (onto the column).

MS conditions: *m/z* 50-750 ESI positive ion mode. In batch 2, samples were injected in full scan (MS only) and multiple reaction mode (MRM). MRM started from 1.5 min, with an isolation mass window of 5 Da, a collision energy of 30 V, and the following list of precursor ions throughout the LC run, running sequentially: *m/z* of 549.4, 324, 504, 310, 417, 338, 387.3, 387.9, and 619. The data files were mass calibrated prior to using the standard algorithms to 'extract compounds' from the chromatogram using both the MS and MS/MS data within a 1 min retention time window.

5.3.6.4 Cosine similarity (dot product score) as a method of comparing the similarity of spectra

Cosine similarity is a numerical score or comparison between two vectors, which is completed for mass spectrometry data by computationally converting the spectra into vectors in n-dimensional space (Neumann and Böcker 2010). These vectors can then be compared using the dot product calculation (Stein and Scott 1994), which by definition includes the cosine angle between the vectors (Yang *et al*. 2013). A cosine score of 1 indicates the two spectra compared are identical and a cosine score of 0 indicates no

similarities between spectra. The edge linking two nodes produced in the final network represents the cosine similarity score with the thickness indicating similarity (Yang *et al*. 2013).

5.3.6.5 Molecular network using Global Natural Products Social Molecular Networking (GNPS)

A molecular network was created using the online workflow at GNPS (GNPS 2015). The data was filtered by removing all MS/MS peaks within +/- 17 Da of the precursor *m/z*. MS/MS spectra were window filtered by choosing only the top 6 peaks in the +/- 50 Da window throughout the spectrum. The data was then clustered with MS-Cluster with a parent mass tolerance of 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da to create consensus spectra, meaning spectra identified as identical were grouped together to form one node. Further, consensus spectra that contained less than one spectrum were discarded. A network was then created where edges were filtered to have a cosine score above 0.65 and more than six matched peaks. Further edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top ten most similar nodes. The spectra in the network were then searched against GNPS's spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks. An example of using this information to generate a network is seen in Figure 5-7.

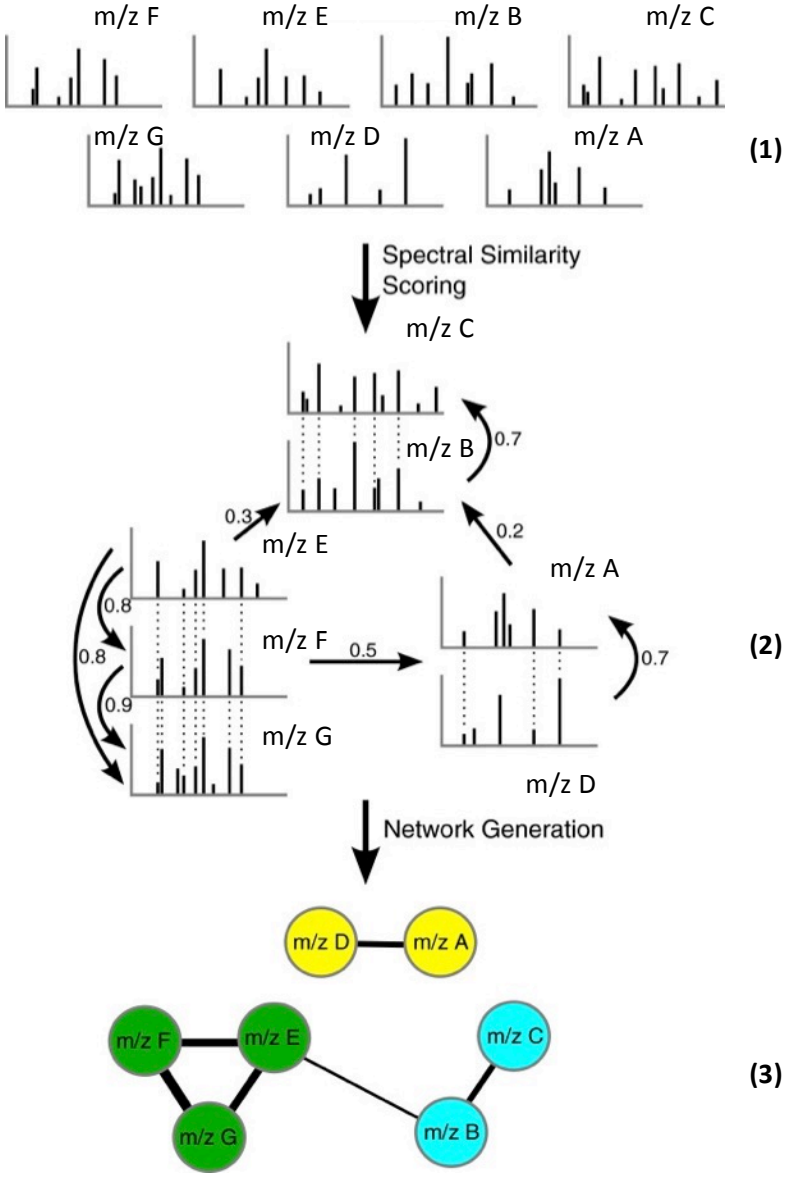


Figure 5-7 Example of using cosine similarity of tandem MS data to generate a molecular network. (1) Spectra generated using HPLC-MS/MS. (2) Similarity of principal peaks of fragmented spectra compared using their cosine similarity (section 5.3.6.4). (3) Molecular network generated using this similarity with each node representing an individual spectra (potentially multiple spectra if determined to be the same) and the edge between them the cosine similarity. Figure modified from (Scheubert *et al*. 2013).

5.4 Results and discussion

5.4.1 Isolation and identification of antibacterial compounds from marine sponges using bioautographic TLC – MS

Antibacterial activity was visualised using direct bioautographic TLC against MRSA (NCTC 11939) followed by identification of the parent masses responsible for activity using TLC-MS. This was completed on all active samples with sufficient material to complete all tests. The results are displayed in Table 5-5.

Table 5-5 Bioautographic TLC - MS. Including the Rf [1]6:4 (hexane : acetone) and [2]8:2 (dichloromethane : methanol), estimation of activity and observed mass value of active compound. Observed isotope patterns and the predicted mass values are also recorded. Predicted mass was estimated by calculating difference in mass that the formation of the selected ion would cause when compared to the observed mass. Predicted masses are coloured based on their classification post dereplication. Black = Known compound matched to sponge, Red = Novel compound, Blue = Possibly novel compound.

Sample	Estimation of activity	Rf of active spot	Observed mass value (m/z)	Predicted mass values			Isotope pattern
				$(M+H)^+$	$(M+Na)^+$	$(2M+Na)^+$	
As10 Crambe crambe[1]	++	0.00	282.3	281.3	259.3	129.7	
	+	0.28	672.5	671.5	649.5	324.8	
	+	0.38	319.2	318.2	296.2	148.1	
			291.2	290.2	268.2	134.1	
	++	0.48	229.2	228.2	206.2	103.1	
			429.3	428.3	406.3	203.2	
As13 Aplysina aerophoba[1]	+++	0.06	261.1	260.1	238.1	119.1	
			305.2	304.2	282.2	141.1	
	++	0.19	251.1	250.1	228.1	114.1	
			409.2	408.2	386.2	193.1	
2w6 Hymeniacidon perleve[1]	+	0.00	253.2	252.2	230.2	115.1	
	+	0.44	327.2	326.2	304.2	152.1	
2w8 Hymeniacidon perleve[1]	+	0.44	327.2	326.2	304.2	152.1	
			454.4	453.4	431.4	215.7	
	+	0.48	415.4	414.4	392.4	196.2	
			909.6	908.6	886.6	443.3	
2w9 Amphilectus fucorum[1]	+	0.38	360.3	359.3	337.3	168.7	
	+	0.56	421.4	420.4	398.4	199.2	
2w14 Halichondria panicea[1]	+	0.04	269.1	268.1	246.1	123.1	
	+	0.21	522.6	521.6	499.6	249.8	
	+	0.31	473.4	472.4	450.4	225.2	
			320.2	319.2	297.2	148.6	
	+	0.41	339.3	338.3	316.3	158.2	
2w15 Halichondria panicea[1]	+	0.43	339.3	338.3	316.3	158.2	
	+	0.50	909.6	908.6	886.6	443.3	
2w18 Halichondria panicea[1]	+	0.25	337.2	336.2	314.2	157.1	
			353.2	352.2	330.2	165.1	
	++	0.38	369.3	368.3	346.3	173.2	

Sample	Estimation of activity	Rf of active spot	Observed mass value (m/z)	Predicted mass values			Isotope pattern
				$(M+H)^+$	$(M+Na)^+$	$(2M+Na)^+$	
2w20 Dysidea fragilis[1]	+	0.06	326.4	325.4	303.4	151.7	
			485.3	484.3	462.3	231.2	
	++	0.23	522.6	521.6	499.6	249.8	
			455.3	454.3	432.3	216.2	
2Jo2 Amphilectus fucorum[1]	+++	0.00	263.2	262.2	240.2	120.1	
			311.2	310.2	288.2	144.1	
As19 Agelas oroides[1]	+	0.44	318.0	317.0	295.0	147.5	Br
			316.0	315.0	293.0	146.5	Br
	+	0.69	385.3	384.3	362.3	181.2	
			469.4	468.4	446.4	223.2	
	+	0.81	291.2	290.2	268.2	134.1	
			329.2	328.2	306.2	153.1	
Ms12 Crambe crambe[2]	++	0.00	400.3	399.3	377.3	188.7	
	++	0.06	241.2	240.2	218.2	109.1	
			248.2	247.2	225.2	112.6	
	+	0.23	479.1	478.1	456.1	228.1	
			493.1	492.1	470.1	235.1	
Ms13 Aplysina aerophoba[2]	+	0.00	365.1	364.1	342.1	171.1	Br_2
			423.1	422.1	400.1	200.1	Br_2
	+	0.08	338.0	337.0	315.0	157.5	Br_2
	+	0.16	522.0	521.0	499.0	249.5	Br_2
	+++	0.75	361.9	360.9	338.9	169.5	Br_2
Ms16 Sarcotragus sp[2]	++	0.50	490.9	489.9	467.9	234.0	Br_2
	+++	0.75	361.9	360.9	338.9	169.5	Br_2
Ms19 Agelas oroides[2]	++	0.26	389.9	388.9	366.9	183.5	Br_2
	++	0.33	340.1	339.1	317.1	158.6	Br
Mf3 Agelas oroides[2]	++	0.29	268.0	267.0	245.0	122.5	Br
			327.0	326.0	304.0	152.0	Br
	++	0.40	313.5	312.5	290.5	145.3	Br_2
			341.0	340.0	318.0	159.0	Br
	+	0.69	290.9	289.9	267.9	134.0	Br_2
	++	0.79	486.4	485.4	463.4	231.7	
			488.4	487.4	465.4	232.7	
Mf6 Agelas oroides[2]	++	0.15	405.9	404.9	382.9	191.5	
			449.3	448.3	426.3	213.2	
	++	0.28	410.0	409.0	387.0	193.5	Br_2
Mf14 Agelas oroides[2]	++	0.00	256.8	255.8	233.8	116.9	
	+	0.35	410.0	409.0	387.0	193.5	Br_2
	++	0.83	437.3	436.3	414.3	207.2	
			481.3	480.3	458.3	229.2	
	+	0.92	409.2	408.2	386.2	193.1	
Mf15 Crambe crambe[2]	+++	0.00	504.4	503.4	481.4	240.7	
	+	0.35	280.2	279.2	257.2	128.6	
			294.2	293.2	271.2	135.6	
Mf16 Crambe crambe[2]	+++	0.00	248.5	247.5	225.5	112.8	
			504.4	503.4	481.4	240.7	
	++	0.20	315.3	313.3	291.3	145.6	
Mf19 Crambe crambe[2]	+	0.20	401.3	400.3	378.3	189.2	
	+	0.35	400.3	399.3	377.3	188.7	
Af3 Agelas oroides[2]	+	0.15	304.3	303.3	281.3	140.7	
	+	0.50	429.1	428.1	406.1	203.1	

The results displayed in Table 5-5 show that in the majority of cases individual compounds of MW less than 550 were responsible for the activity identified in the overlay assay and thus most likely the activity displayed in the MIC tests (section 4.4.6). From these 86 active spots around 20% of samples contained brominated compounds (determined by isotope splitting), all of which were from sponges of Greek origin.

5.4.1.1 Comparison of MarinLit and Scifinder for data mining and dereplication

The resolving factor of *MarinLit* (without any refinements) and *SciFinder* (multiple refinements) was then directly compared for all predicted masses, some examples of each predicted ion are shown in Table 5-6, Table 5-7 and Table 5-8.

Table 5-6 Number of matched MW compound matches, in *MarinLit* and *SciFinder* for selected masses if the observed peak was *m/z* = $(M + H)^+$.

Sponge Species	Predicted mass if observed ion = $(M+H)^+$	Number of database matches obtained			
		MarinLit (by MW only)	*SciFinder*		
			By MW	Refined by 'natural product'	Refined by 'natural product' and 'marine'
As10 C. crambe	281.26	4	114032	494	63
Mf15 C. crambe	503.36	6	25347	61	9
2w8 H. perleve	326.20	56	46335	323	30
Ms16 Sarcotragus. sp	489.94	3	25307	18	0

Table 5-7 Number of matched MW compound matches, in *MarinLit* and *SciFinder* for selected masses if the observed peak was *m/z* = $(M + Na)^+$.

Sponge Species	Predicted mass if observed ion = $(M+Na)^+$	Number of database matches obtained			
		MarinLit (by MW only)	*SciFinder*		
			By MW	Refined by 'natural product'	Refined by 'natural product' and 'marine'
Mf19 C. crambe	378.32	56	90825	257	40
As13 A. aerophoba	238.14	60	27728	409	54
2w6 H. perleve	304.20	231	42884	188	28
Mf6 A. oroides	382.90	4	28315	46	9

Table 5-8 Number of matched MW compound matches, in *MarinLit* and *SciFinder* for selected masses if the observed peak was *m/z* = (2M + Na)$^+$.

Sponge Species	Predicted mass if observed ion= (2M+Na)$^+$	Number of database matches obtained			
		MarinLit (by MW only)	*SciFinder*		
			By MW	Refined by 'natural product'	Refined by 'natural product' and 'marine'
2w6 H. perleve	378.32	56	90825	257	40
2w15 H. panicea	238.14	60	27728	409	54
2u2 Unknown	304.20	231	42884	188	28

These results showed that *MarinLit* was concise and focused solely on marine natural products and produced similar matches to refined *SciFinder* results. *Scifinder* did on some occasions seemingly narrow the field down further than *MarinLit,* For example, in Table 5-5; 2w6 produced 231 results in *MarinLit* and only 28 in *SciFinder*. However problems have been reported with the refinement of parameters in *SciFinder* with multiple phrase frequency and proximity analysis needed to collect a full list of results (Kostoff *et al*. 2000). Therefore, any discrepancies found after refinement in *SciFinder* does not dismiss the validity of the data retrieved by *MarinLit*. This comparison confirmed that *MarinLit* was highly efficient in retrieving marine natural product data.

5.4.1.2 Novel and potentially novel compounds

The predicted ion mass of 23 compounds met the criteria of possibly novel within the parameters defined and 46 compounds (or masses) were considered to be novel. The most promising of these leads were the eight parent masses where no molecular weight matches occurred for any of the expected ions. These were two parent masses from 2w14 (*H. panicea*), 2w20 (*D. fragilis*) and Ms13 (*A. aerophoba*) and single parent masses from Mf14 (*A. oroides*) and Mf16 (*C. crambe*). The majority of the molecular weights identified were also less than 550.

Due to the enormous number of identified natural products already reported, the difficulty in the discovery of novel bioactive compounds was raised in a report by Corley and Durley and among their suggestions of commercially available databases to assist this was the use of the *MarinLit* database (Corley and Durley 1994).

One conceivable flaw in the potential novelty of the compounds discovered is that it was based on many assumptions; one of which is that the samples were directly isolated from the sponge and will therefore show up when searching a marine database. It was possible

that some of the masses could have originated from contamination, which is possible all the way through the process. For this reason, the results were also compared to those from previous extracts and common contaminants and no obvious matches were present. All samples were very carefully run individually using the TLC-MS interface and many of the contaminants, such as plasticisers, were recognised and disregarded during the run.

5.4.1.3 Refinement of results

5.4.1.3.1 Identifying 'drug-like' molecules found in marine sponges using *MarinLit*

A search was completed using *MarinLit* to show how refinement by the criteria identified (section 5.3.4.2) reduced the number of lead compounds previously identified from marine sponges (Table 5-9).

Table 5-9 Number of matches for all sponge extracts studied at each step of compound refinement using *MarinLit.*

Stage of refinement	Total number of matches found in MarinLit
Before refinement (MW only)	>2000
Taxonomy	1244
MS data	987
Lipinski Rule of Five	299

From all samples studied, compounds from *Agelas oroides* showed the most results following refinement, providing the most potential 'drug-like' leads. Stepping through the process, 535 compounds were suggested post taxonomic refinement; 377 post-MS refinement and 110 had drug-like properties. For *Sarcotragus* species no compounds extracted fitted the criteria as being drug-like post refinement.

5.4.1.3.2 Refinement of database leads by isotopic patterns displayed in the mass spectrum

The active compound found at Rf 0.29 (8:2, dichloromethane : methanol) in extract Mf3 (*Agelas oroides*) clearly showed two equal intensity peaks at *m/z* 267.0 and 269.0 (M and M+2), which indicated the presence of a single bromine group within the compound (Figure 5-8). The average isotopic peak was recorded at *m/z* 268.0.

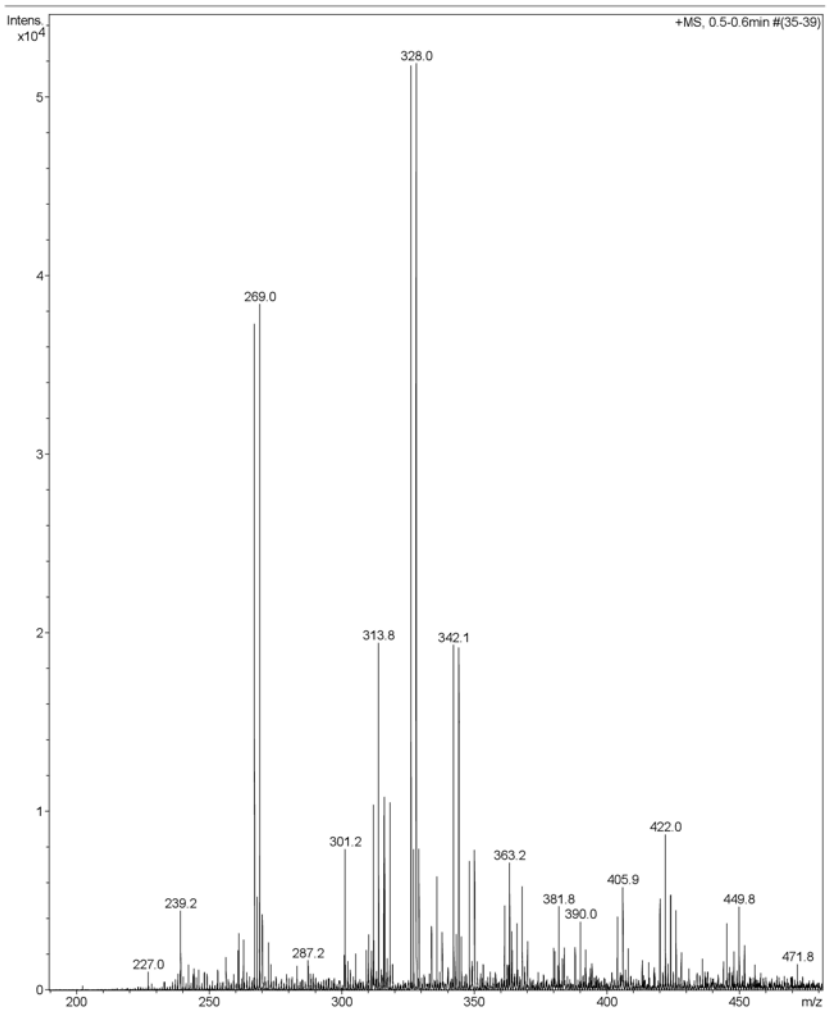


Figure 5-8 Mass spectrum of active spot observed in bioautographic overlay assay extract Mf3 (*A. oroides*). Two monobrominated principal peaks detected at *m/z* 269.0 and *m/z* 328.0.

Refinement was completed using *MarinLit* and this isotopic information, which led to the removal of any products not containing a single bromine, proposed potential structures as depicted in Figure 5-9.

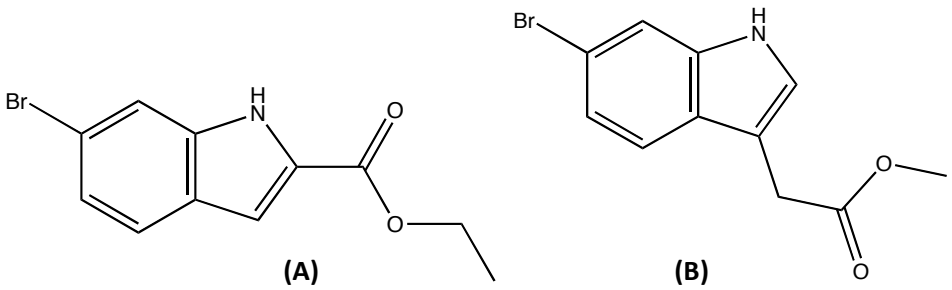


Figure 5-9 Potential structure of bioactive compound found in extract Mf3 (*A. oroides*). (A) Ethyl 6-bromo-2-indol-carboxylate and (B) Methyl 6-bromoindolyl-3-acetate, *m/z* 267.

Another example of brominated peaks was seen in extract Ms19 (*A. oroides*). The principal and average isotopic peak seen in Figure 5-10 is *m/z* 389.95 but as the compound appears dibrominated, the monoisotopic mass would be *m/z* 387.95. This would make the predicted monoisotopic mass 386.95 if an $(M + H)^+$ ion was observed. Upon refinement by taxonomy and the isotopic information gathered, *MarinLit* identified this active compound as oroidin, the structure of which is shown in Table 5-3; detailing a list of standards used for the HPLC-MS/MS formed molecular network. This would be the first time oroidin has been shown to display direct activity against the clinically relevant resistant bacteria MRSA (NCTC 11939), although a recent study demonstrated antibacterial activity against other Gram-positive strains (Zidar *et al*. 2014) and other studies have determined its ability to inhibit biofilm formation (Hodnik *et al*. 2014; Stowe *et al*. 2011).

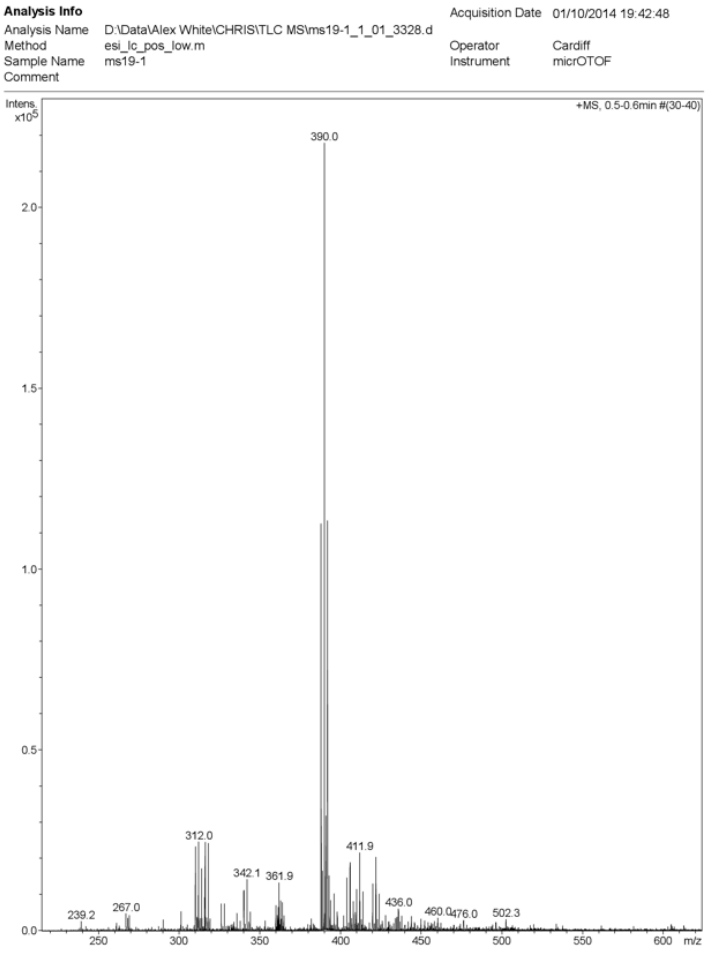


Figure 5-10 Mass spectrum of active spot observed in bioautographic overlay assay extract Ms19 (*A. oroides*), average isotopic peak was detected at *m/z* 390.0. An Isotope pattern consistent with two bromine atoms was observed.

Another example of identifying a brominated peak was in extract Ms13 (*A. aerophoba*), where it was originally thought that aeroplysinin had been isolated. The M + 2 peak appeared at *m/z* 338.0, which when compared to *MarinLit* suggested aeroplysinin, which would have a monoisotopic peak at *m/z* 337.9 $(M+H)^+$. However the mass values found in *MarinLit* are based on the monoisotopic mass, which for this sample was found at *m/z* 336.0, as the sample appeared dibrominated (Figure 5-11). This compound therefore did not match aeroplysinin and had a suggested molecular weight of 335.0 assuming the peak found was $(M+H)^+$.

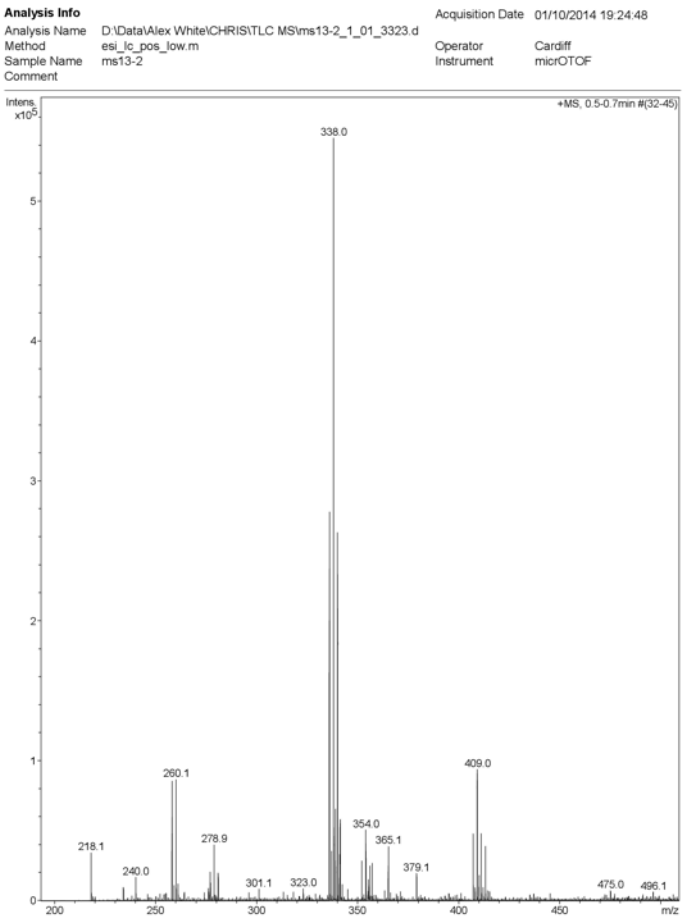


Figure 5-11 Mass spectrum of active spot observed in bioautographic overlay assay extract Ms13 (*A. aerophoba*) with clear average isotopic peak detected at m/z 338.0. Dibrominated pattern was also observed.

On searching *MarinLit*, no records were found from the genus *Aplysina* for the potential monoisotopic mass of 335.0. This would suggest that this was a potentially novel compound perhaps similar to aeroplysinin. There was a possibility that this compound was formed due to the oxidation of aeroplysinin following increased exposure to oxygen on removal from water and drying in the atmosphere in Greece (Figure 5-12). It was noted in section 4.4.1.2

that *A. aerophoba* was bright yellow in the water and turned brown instantly on removal, probably due to oxidation of a yellow compound within the sponge. Aeroplysinin is well known to be bright yellow compound with a colour comparable to that of vitamin E (Abou-Shoer *et al*. 2008). The corresponding active keto-alcohol, potentially isolated in this study, has been synthesised for use as a precursor in the synthesis of aeroplysinin using a reduction reaction (Andersen and Faulkner 1975) but has never been extracted directly from a sample from the genus *Agelas*.

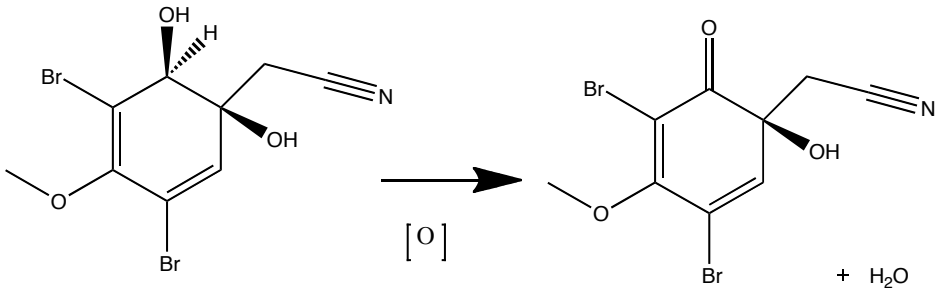


Figure 5-12 Potential oxidation on exposure to air of aeroplysinin to form (S)-2-(3,5-dibromo-1-hydroxy-4-methoxy-6-oxocyclohexa-2,4-dien-1-yl)acetonitrile.

Analysis of the halogenated MS data was useful for refinement of species containing halogens. However, this did not apply to approximately 80% of the species investigated, with none of the active compounds identified in the Welsh sponge samples showing any obvious halogenation.

5.4.1.4 TLC-MS analysis of an unidentified sponge

5.4.1.4.1 Identification of sponge species and differences in preparation

An unknown sponge sample collected from Greece was identified by Jennifer Jones (independent consultant), using microscopy, as being from the family *Geodiidae*, which is of the same family as *Pachymatisma johnstonia* (elephant hide sponge), found in UK waters. The sample was identified as the genus *Geodia* but could not be classified to species level and was considered to be potentially novel. It displayed unusually large spicules that reached a few mm long, which protruded from the samples like slivers of glass (silica) and produced a stinging sensation similar to nettles upon touch. The stinging sensation was noted upon collection of the sample. The sample also displayed an unusual encrusting layer that visually looked like algae (Figure 5-13). Therefore, for this particular sample, the crust of the sponge was separated from the bulk of material and tested separately as it is known that spatial

differences in metabolite production within the sponges tissues can alter the sponges chemical characteristics (Esquenazi *et al*. 2008). The core of the sponge and the crust of the sponge were labelled u2 and u3, respectively. A cross sectional area of the sponge labelled u1 was not tested in this study.

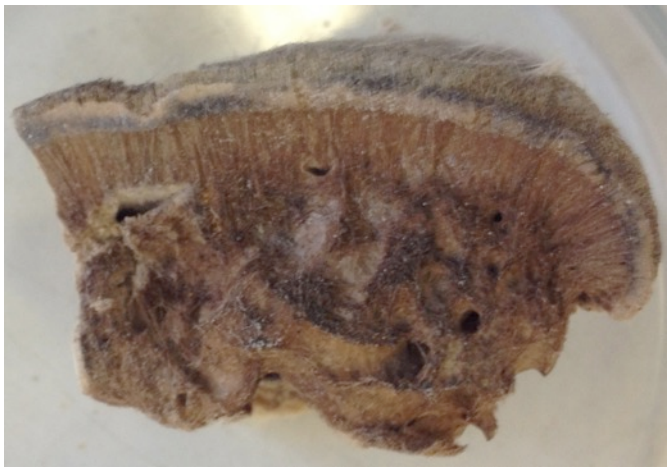


Figure 5-13 Digital photograph of a cross section of a sponge sample of the *Geodia sp*. considered to be potentially novel. Large spicules and crust can be observed at the top of the sponge.

The sponge was cross-sectioned, extracted using the sequential extraction method (section 2.2.3), and screened for antibacterial activity using bioautographic overlay TLC technique (Table 5-10).

Table 5-10 Bioautographic TLC – MS of *Geodia sp*. Including the Rf 6:4 (hexane : acetone) and estimation of activity and observed mass value of active compound. Observed isotope patterns and the predicted mass values are also recorded. Predicted mass was estimated by calculating difference in mass that the formation of the selected ion would cause when compared to the observed mass. Predicted masses are coloured based on their classification post dereplication. Black = Known compound matched to sponge, Red = Novel compound, Blue = Possibly novel compound.

Sample	Estimate of activity	Rf of active spot	Observed mass value (m/z)	Predicted mass values			Isotope pattern
				$(M+H)^+$	$(M+Na)^+$	$(2M+Na)^+$	
2u2 Unknown	+	0.28	394.3	393.3	371.3	185.7	
	++	0.35	293.2	292.2	270.2	135.1	
			307.2	306.2	284.2	142.1	
	++	0.41	765.7	764.7	742.7	371.4	
	+	0.90	685.5	684.5	662.5	331.3	
2u3 Unknown	+	0.31	376.3	375.3	353.3	176.7	
	+	0.44	443.4	442.4	420.4	210.2	
			586.4	585.4	563.4	281.7	

Eight different parent masses with antibacterial activity were identified using TLC-MS combined with a bioautographic overlay assay (Table 5-5). No matches in masses were detected between the centre of the sponge and the crust. Upon comparing the potential ionization results in *MarinLit*, only one hit occurred when limiting the search by taxonomical Genus. The biologically active extract, obtained from the inside of the sponge had an observed *m/z* of 685.5 with the predicted mass of 662.5 $(M + Na)^+$, was provisionally identified as eryloside-T (Figure 5-14)(Afiyatullov *et al*. 2007).

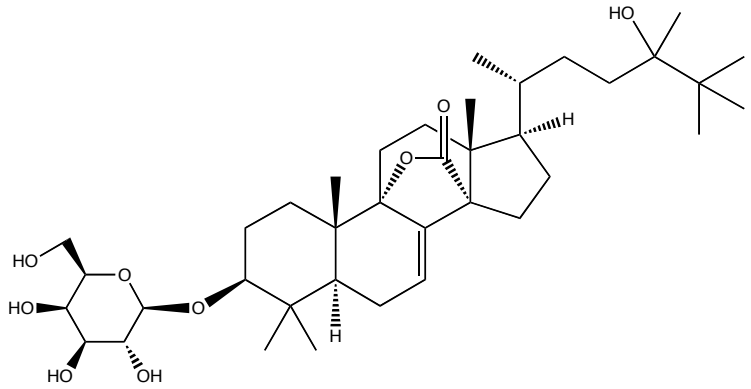


Figure 5-14 Chemical structure of erloside-T. (Formula: $C_{38}H_{62}O_9$; Exact Mass: 662.43940).

No other matches occurred when searching *MarinLit* for known compounds, therefore the other seven biologically active extracts were considered to be potentially novel compounds based on their observed *m/z* measurements as no matches occurred with the sponge Genus. Four of the predicted mass values showed no matches to any compounds isolated from marine sources and therefore were considered novel masses by the defined parameters of this project. A larger proportion of potentially novel compounds were identified from this unknown sample (79 %) when compared to the rest of the samples studied (10 %), this is most likely because the Genus is not as well studied as some of the other sponges shown in Table 5-5.

In summary, TLC-MS has proved a useful method of lead refinement identifying multiple compounds that are potentially novel to marine natural products. It has also tentatively identified multiple known compounds expected to be found in marine sponges, although non are proven. This method did however have some deficiencies, the main of which was that the only information used for identification was parent mass and many compounds could potentially be discarded due to a parent mass match when they were indeed novel. A more thorough approach would be to use TLC-MS as a primary method of parent mass

identification. This could then be followed by another hyphenated technique to gain more structural information such as HPLC-NMR or HPLC-MS/MS. Another method could be to use more sophisticated MS techniques coupled with the TLC-MS hardware, such as accurate MS or MS/MS. This would generate more detailed information directly from the TLC plate without need for further separation techniques. The potential advances in coupled MS techniques or suggestion of further techniques post TLC-MS in no way discounts the use of this method in the project, together with bioautographic TLC, as part of the workflow of natural product drug discovery.

5.4.2 High performance counter current chromatography as a method for separation of antibacterial compounds from marine sponges

5.4.2.1 Method development

Initial method development focused on the Greek sponge *Agelas oroides*, which was found in the nets of fisherman near the Island of Samos, Greece. Simple separation with TLC and identification with MS and NMR resulted in the isolation of multiple known compounds including plasticiser contaminants, cholesterol and clionasterol (section 3.4). Some antimicrobial brominated compounds were also extracted but full structure elucidation proved impossible.

A gradient HPCCC run was completed, on a methanol extract of the same sponge samples, in an attempt to retrieve these compounds in a greater yield. Post HPCCC separation, all UV visible compounds or mixtures were concentrated using a centrifugal concentrator and fractions were further refined by the quantity of material needed to characterise any isolated compounds and those showing the clearest peaks on the UV spectra (Figure 5-15). This further refinement resulted in 9 fractions (14, 15, 16, 20, 26, 38, 60, 121 and 122), which were then tested for antibacterial activity using bioautographic TLC against MSSA (Figure 5-16), and the active fractions (38 and 60) were sent to the national mass spectrometry facility in Swansea for accurate MS and prediction of the molecular formula.

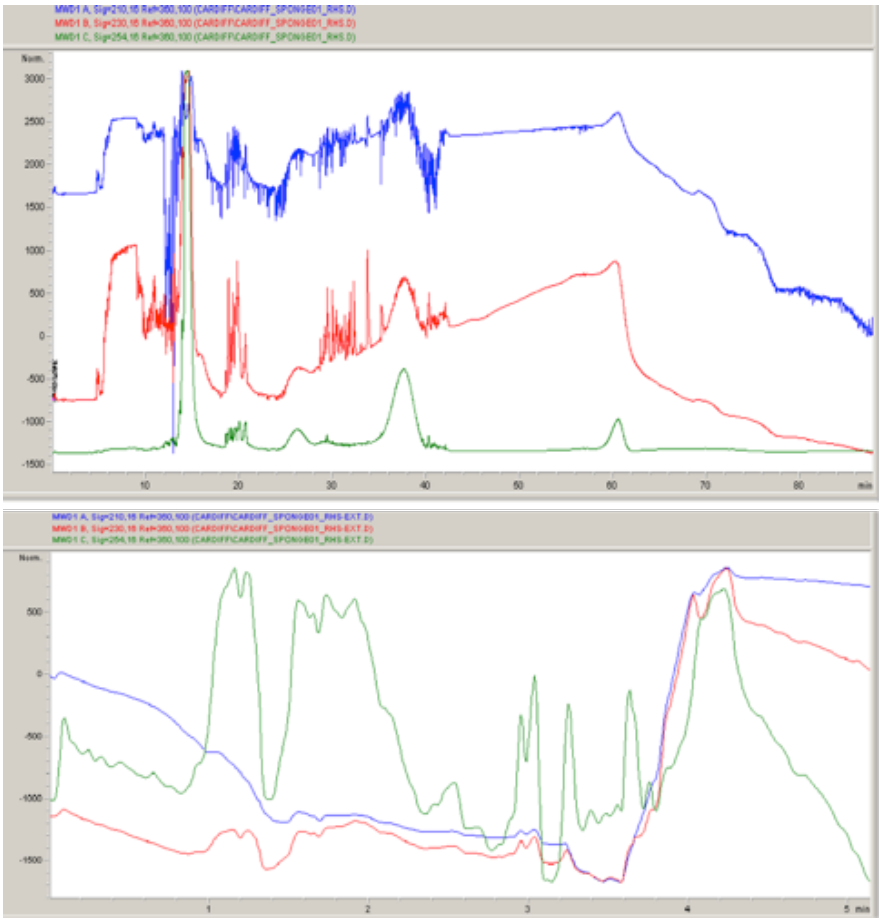


Figure 5-15 UV spectra following HPCCC separation of *Agelas oroides* recorded at 210nm, 230nm and 235nm displayed in blue, red and green respectively. (A) Primary separation and (B) Extrusion.

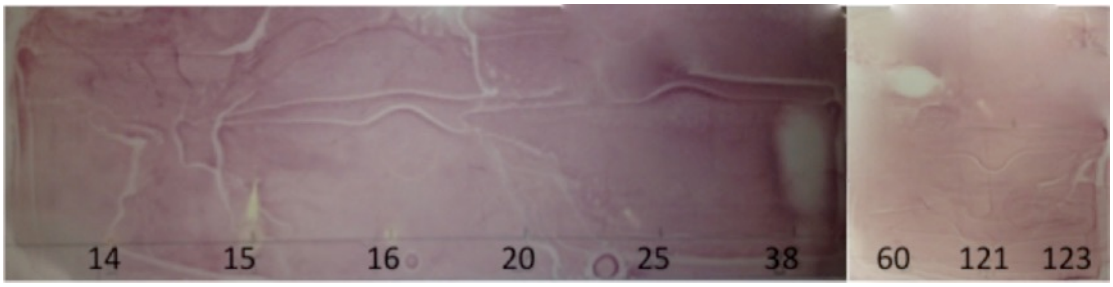


Figure 5-16 Digital photograph of bioautographic overlay assay of *Agelas oroides* fractions separated during the method development HPCCC run. TLC eluted with 6:4 (hexane : acetone) and activity visualised against MSSA. Clear activity found in fraction 38 and 60.

5.4.2.1.1 Identification of antibacterial fractions separated using method development HPCCC

Accurate mass spectrometry analysis of fraction 38 and 60 revealed brominated alkaloids as the compounds responsible for the antibacterial activity against MSSA displayed in Figure 5-16.

Accurate mass spectrometry (C, Figure 5-17) of fraction 38 identified a single compound with *a m/z* of 267.8603 $(M + H)^+$, and molecular formula of $C_5H_4NO_2Br_2$, suggesting 4,5-dibromo-1H-pyrrole-2-carboxylic acid (A, Figure 5-18), which is a biosynthetic precursor to oroidin, an active metabolite often found in marine sponges (Pinder 1989).

Mass spectrometry (A and B, Figure 5-17) of fraction 60 identified two compounds. The first with *m/z* $(M + H)^+$ of 223.8705 and molecular formula of $C_4H_4NBr_2$, which suggested the most likely structure of 2,3-dibromo-1H-pyrrole (B, Figure 5-18), also a precursor to oroidin (Pinder 1989). The second compound with *m/z* of 248.8658 $(M + H)^+$ and molecular formula of $C_5H_2N_2Br_2$ suggested the most likely structure of 4,5-dibromo-1H-pyrrole-2-carbonitrile (C, Figure 5-17), also an oroidin precursor previously extracted from the methanol extract of *Agelas oroides* (Forenza *et al*. 1971).

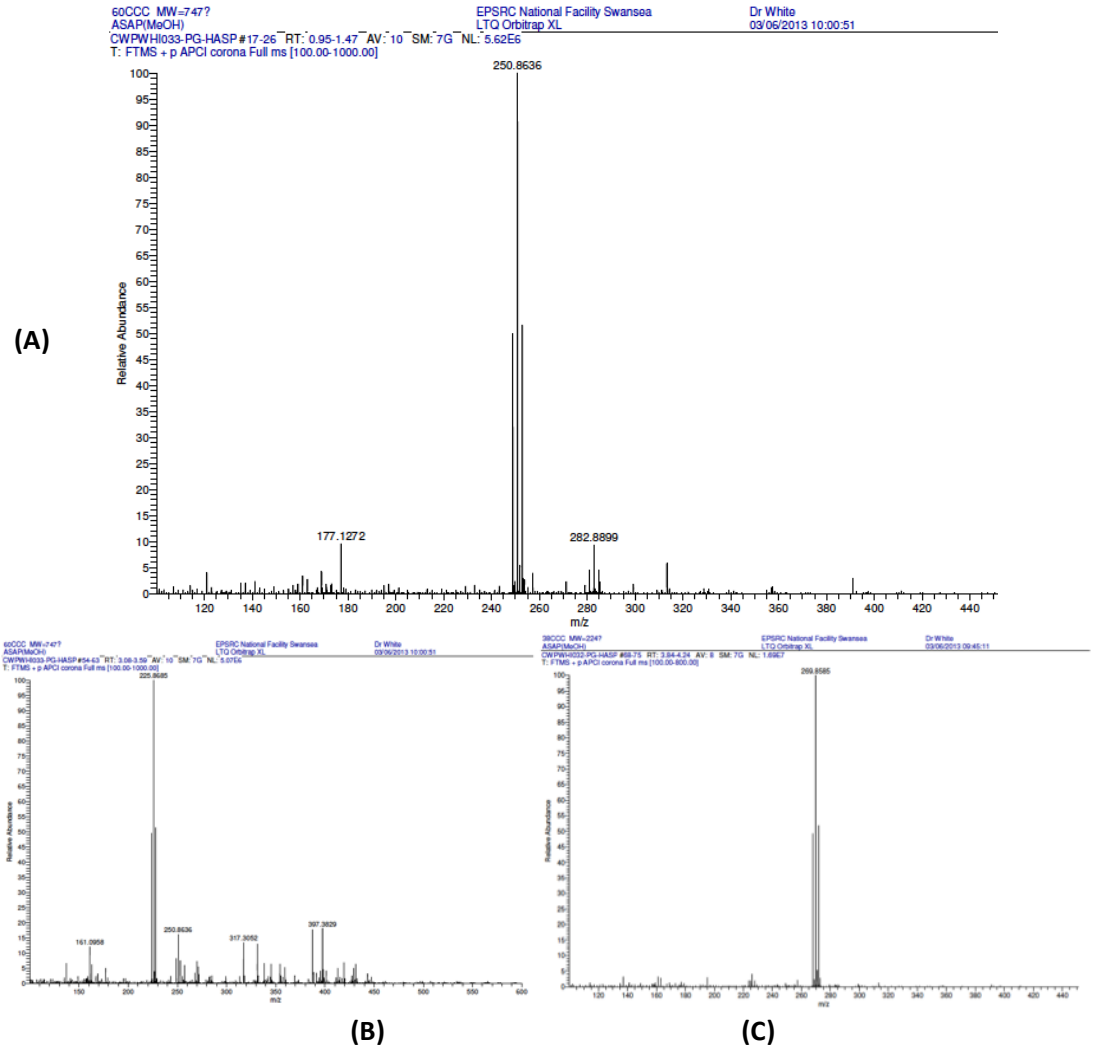


Figure 5-17 Mass spectrometry analysis of active antibacterial fractions of methanol extract of *Agelas oroides* separated via development HPCCC run. (A) and (B) Spectra of two compounds observed in fraction 60, both of which displayed isotope patterns consistent with that of two bromines; (C) Spectra of fraction 38, isotope pattern observed consistent of that of two bromines.

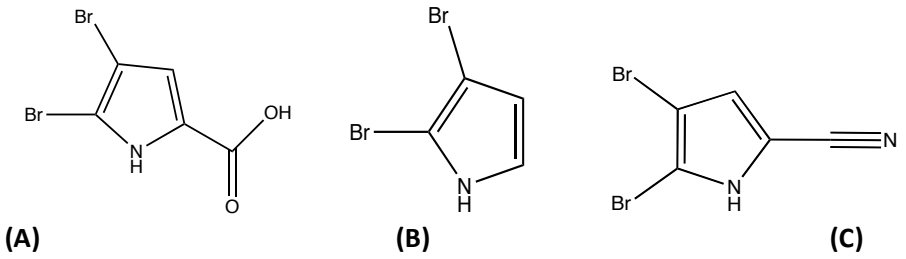


Figure 5-18 Structure of (A) 4,5-dibromo-1H-pyrrole-2-carboxylic acid. (B) 2,3-dibromo-1H-pyrrole. (C) 4,5-dibromo-1H-pyrrole-2-carbonitrile.

One of the masses from fraction 38 (267.8603 (M + H)$^+$) matched one of those originally isolated using TLC (section 3.4.2.6), confirmed with repeat MS, but on this occasion the molecular formula was confirmed due to the use of accurate MS. The use of HPCCC in this

preliminary test succeeded in separating active compounds from inactive material in a greater yield than the previous methods with fraction 38, where a 6% yield was achieved compared to 0.4% for a compound with the same mass separated using TLC. This shows that HPCCC was an excellent method of separating compounds with little or no loss of material (Sutherland and Fisher 2009). A limitation of this initial separation using HPCCC was that only UV active compounds were tested for activity. Therefore this method may have missed any potential antibacterial products, which were not heavily saturated and therefore did not produce a UV signal. A further limitation was that TLCs were completed prior to the HPCCC run to confirm solubility within the phases but no phase transition was observed in active products. In practice this meant that the solvent selection relied on luck to provide good separation rather than being activity based.

5.4.2.2 Antibacterial activity based solvent system selection

The preliminary HPCCC run (section 5.4.2.1) relied on serendipity for isolation of active material and while this proved successful in isolating some active compounds, it was decided that a template solvent system that could be used to separate any active marine extracts should be developed. Template solvent systems were developed based upon the partitioning and solubility of the compounds, which were deemed active from the bioautographic, overlay results. Both acetone and methanol extracts showed good activity in the bioautographic technique but due to the difference in polarity between the compounds found in both, separate solvent systems were required to provide good separation. Three different acetone extracts and two different methanol extracts were chosen to develop the template solvent system. As there was varying partitioning between the chosen extracts when tested using this method, the solvent system chosen was a gradient between when the active compounds switched between phases. Acetone soluble sponge extracts showed clear phase separation using a reverse phase gradient HEMWat (1 : 1 : 1 : 1) to (19 : 1 : 19 : 1). Methanol extracts showed clear phase separation using a normal phase gradient from ethyl acetate-water to butanol-water. The HEMWat scale was utilized as it always has four solvents in each system, which meant that fine adjustments to provide the best solvent system were possible.

5.4.2.3 HPCCC separation

The three acetone; 2w20 (*Dysidea fragilis*), As10 (*Crambe crambe*), 2w18 (*Halichondria panicea*); and two methanol extracts; Ms19 (*Agelas oroides*), 3w18 (*Halichondria panicea*);

used to develop the solvent systems were then separated using HPCCC. It took 7 min and 9 min to reach equilibrium, for the acetone and methanol template systems respectively, before the linear gradient was started.

5.4.2.4 Combination of fractions

UV spectra analysis during CCC separation (Figure 5-19) and post-separation TLC analysis indicated good separation had occurred for all extracts with large colour variations visible in a large proportion of fractions (Figure 5-20). After separation of each extract using HPCCC, fractions deemed active with similar Rf via bioautographic overlay assay against MSSA were combined to give the following fractions (Figure 5-20).

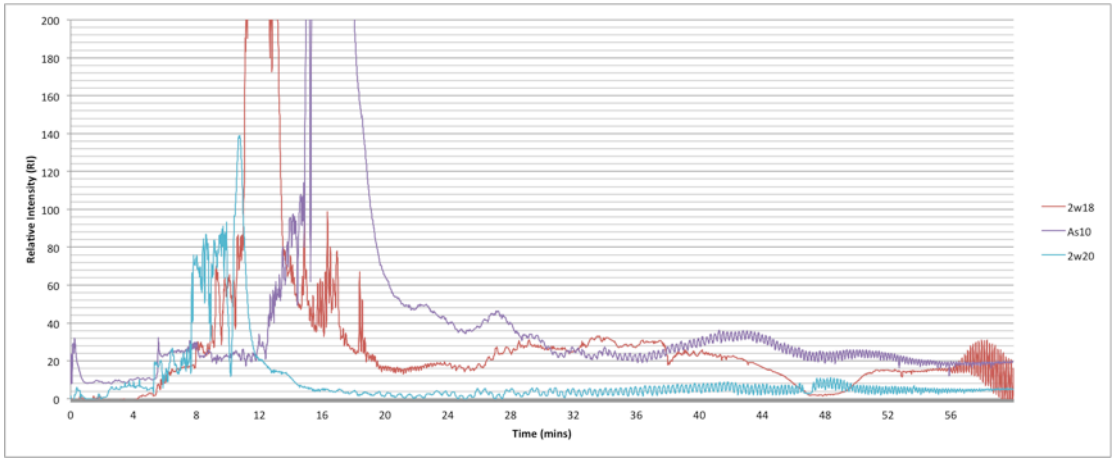
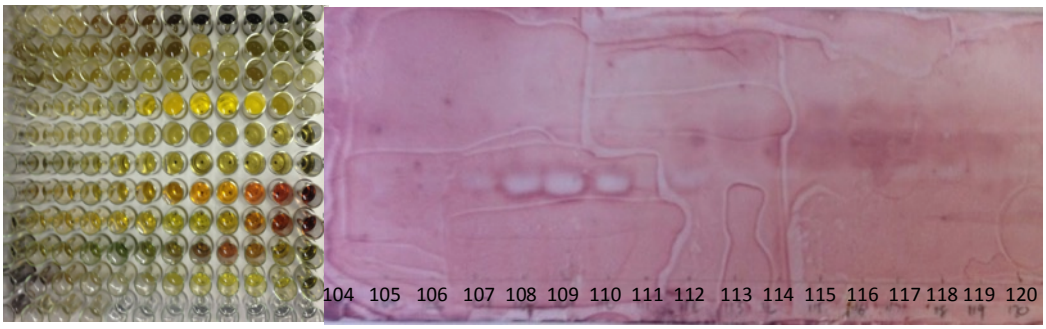


Figure 5-19 UV (254nm) chromatogram of primary separation of acetone extracts 2w20 (*Dysidea fragilis*), As10 (*Crambe crambe*) and 2w18 (*Halichondria panicea*) using HPCCC.



(A) **(B)**

Figure 5-20 (A) Fractions from extract 2w18 separated using HPCCC. (B) Fractions 104 to 120 from HPCCC separation of extract 2w18. Antimicrobial activity was visualised in vials 107-110 using bioautographic technique against MSSA, separated using 4:6 (acetone : hexane), which were combined to form fraction 2w18.6.

Table 5-11 Summary of antibacterial fractions post CCC separation. The number preceding each range of vials combined represents the label assigned to the combined fraction. For example, activity was found in vials 107-110 (in extract 2w18), which were combined to form fraction 2w18.6 as depicted in Figure 5-20.

Fraction	Active vials combined to form fractions
2w20	1) 83-88, 2) 91-94, 3) 101-105, 4) 111-116
As10	1) 21-23, 2) 67-69, 3) 114-117
2w18	1) 14-18, 2) 32-33, 3) 42-45, 4) 70-71, 5) 76-83, 6) 107-110
Ms19	1) 9-12, 2) 13-16, 3) 21-23, 4) 37-39
3w18	1) 25-35, 2) 108-109

5.4.2.5 Antimicrobial activity of HPCCC separated fractions by determining their minimum inhibitory concentration (MIC) and minimum bactericidal concentration testing (MBC) against MRSA and E. coli

The combined fractions were then tested for activity using the broth dilution method (section 4.3.4) and both MIC and MBC were recorded (Figure 5-21).

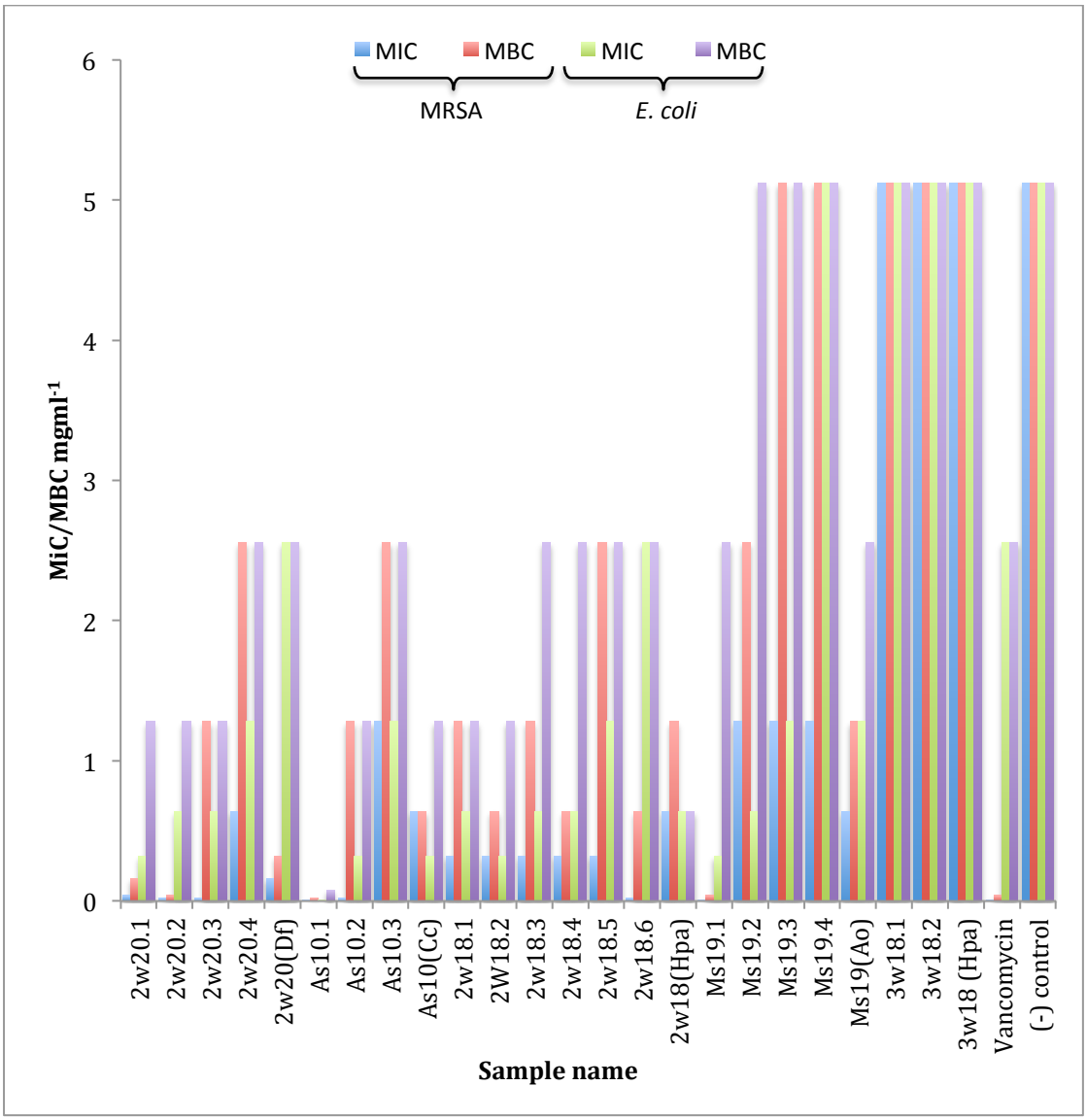


Figure 5-21 MIC and MBC of Greek and Welsh sponge fractions separated via HPCCC against MRSA and *E. coli* using the microdilution method. Parent sponge antibacterial activity also included. Each result was completed in triplicate. (±) = Positive (vancomycin) and negative control = no bacteria.. Species abbreviations: Hpa = *H. panicea*, Df = *D. fragilis,* Cc = *C. Crambe* and Ao = *A. oroides*. No activity observed is indicated by a value = 5.12 mg mL^{-1} (Double concentration of first well).

The MBC provides the most useful information regarding any extract activity as when compared to the MIC, it shows whether an extract is deemed to be bactericidal. The greatest activity of any sample was that of As10.1; this displayed better activity against MRSA and *E. coli* than any other sample or the control vancomycin. In all cases where activity was observed in the parent extract at least one fraction (2w20.1, As10.1, 2w18.2, Ms19.1) showed greater activity against MRSA and *E. coli* than their parent extract. This confirms the

success of the template HPCCC method, as it successfully separated the active material within each extract from the inactive material.

5.4.2.6 Compound identification and dereplication using TLC-MS and parent mass

Direct TLC-MS was then completed on the active samples with sufficient material post MIC testing (Table 5-12) and some examples of the comparison of the predicted mass values of active fractions to *MarinLit* and *SciFinder* are shown in Table 5-13.

Table 5-12 Bioautographic TLC – MS of HPCCC fractions with Rf values following TLC elution with [1]6:4 (hexane : acetone) and [2]8:2 (dichloromethane : methanol), estimation of intensity of antimicrobial activity and observed mass value of active compound. Observed isotope patterns and the predicted mass values were also recorded. Predicted mass was estimated by calculating the difference in mass that the formation of a selected ion would cause when compared to the observed mass. Predicted masses are coloured based on their classification post dereplication. Black = Known compound matched to sponge, Red = Novel compound, Blue = Possibly novel compound.

Sample	Estimation of activity	Rf of Active spot	Observed mass value (m/z)	Predicted mass values			Isotope pattern
				$(M+H)^+$	$(M+Na)^+$	$(2M+Na)^+$	
As10.1 Crambe crambe[1]	+++	0	282.3	281.3	259.3	129.7	
			241.3	240.3	218.3	109.2	
			358.3	357.3	335.3	167.7	
			481.4	480.4	458.4	229.2	
	++	0.13	241.2	240.2	218.2	109.1	
			360.3	359.3	337.3	168.7	
As10.3 Crambe crambe[1]	+	0.13	241.3	240.3	218.3	109.2	
			482.4	481.4	459.4	229.7	$(2M+H^+)$
Ms19.1 Agelas oroides[2]	++	0.84	288.9	287.9	265.9	133.0	Halogen
			290.8	289.8	267.8	133.9	Halogen
			388.9	387.9	386.9	183.5	Halogen
	+++	0.95	288.9	287.9	265.9	133.0	Br_2
			290.9	289.9	267.9	134.0	Br_2
			318.9	317.9	295.9	148.0	
2w18.1 Halichondria panicea[1]	+	0	430.9	429.9	407.9	204.0	
	+	0.25	293.5	292.5	270.5	135.3	
2w18.3 Halichondria panicea[1]	+	0.13	241.2	240.2	218.2	109.1	
			481.4	480.4	458.4	229.2	
			401.3	400.3	378.3	189.2	
2w18.6 Halichondria panicea[1]	+	0.13	403.3	402.3	380.3	190.2	
	+	0.53	411.4	410.4	388.4	194.2	

Table 5-13 Number of database matches from *MarinLit* and *SciFinder* for selected ions *m/z* = $(M + H)^+$ of marine sponge extract fractions following HPCCC and TLC-MS. Rf also displayed 6:4 (hexane : acetone).

Sponge Species	Rf	Predicted mass if observed ion= $(M+H)^+$	Number of database matches obtained			
			MarinLit (by MW only)	*SciFinder*		
				By MW	Refined by 'natural product'	Refined by 'natural product' and 'marine'
As10.1 Crambe crambe	0	281.3	4	114032	494	63
		240.3	2	134803	584	99
		357.3	1	76808	356	42
		480.4	30	44868	141	28
	0.13	240.2	14	75726	454	75
		359.3	7	49608	221	22
As10.3 Crambe crambe	0.13	240.3	2	134899	583	99
		481.4	6	46806	121	10
2w18.3 Halichondria panicea	0.13	240.2	14	75726	454	75
		480.4	30	44868	141	28

Analysing the data show in Table 5-13 was particularly noteworthy with the initial belief that a dimer of the peak appearing at m/z 241.3 may be being formed at m/z 481.4 therefore representing (2M), consequently the actual mass would be 240.3 if $(M + H)^+$ and 481.4 was $(2M + H)^+$. These matching peaks were observed in fractions As10.1, As10.3 and 2w18.3, where similar Rfs in the same elution solvent were detected. However upon searching *MarinLit* and limiting by species, the mass of 480.4 $(M+H)^+$ was frequently detected in *Crambe crambe* and likely relates to either Crambescin B or Crambescin C1 (Figure 5-22).

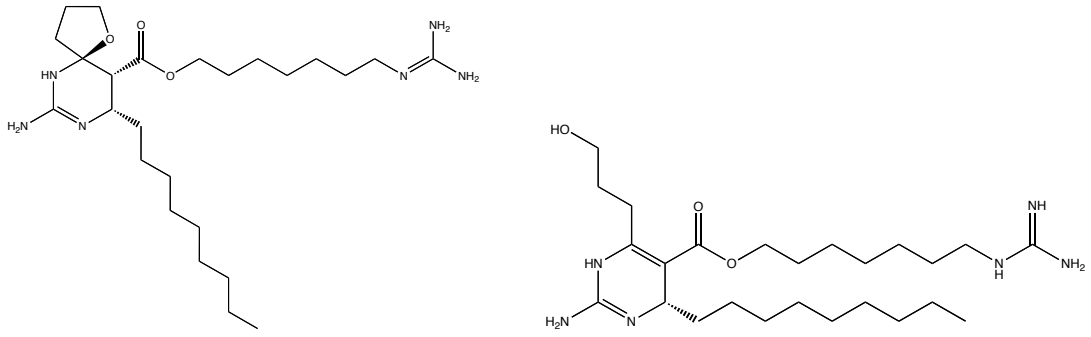


Figure 5-22 Chemical structure of (A) Crambescin B and (B) Crambescin C1, known compounds previously isolated from *C. crambe*. (Mass: 480.3788).

This led to careful examination of the results recorded for fraction 2w18.3 and its mass spectrometry analysis (Figure 5-23). It was clear that this mass spectrum contains a mix of compounds with clear peaks of 240.3 and 480.4 but also a visible peak of 401.3, whereas

fraction As10.3 produced particularly clear peaks. For this reason, the fraction was referred back to the crude sample (Table 5-5) the active masses here showed no matches for 240.3 or 480.4. The workflow was also analysed and the samples were analysed one after another in the MS workflow, suggesting that the appearance of these masses was most likely contaminant from the previous run of *C. crambe* samples. However, this is impossible to confirm with an automatic system, as only principal peaks would have been identified therefore indicating that the same product was present in both samples. Washes were run in between each sample and any such discrepancies were recorded to minimise risk.

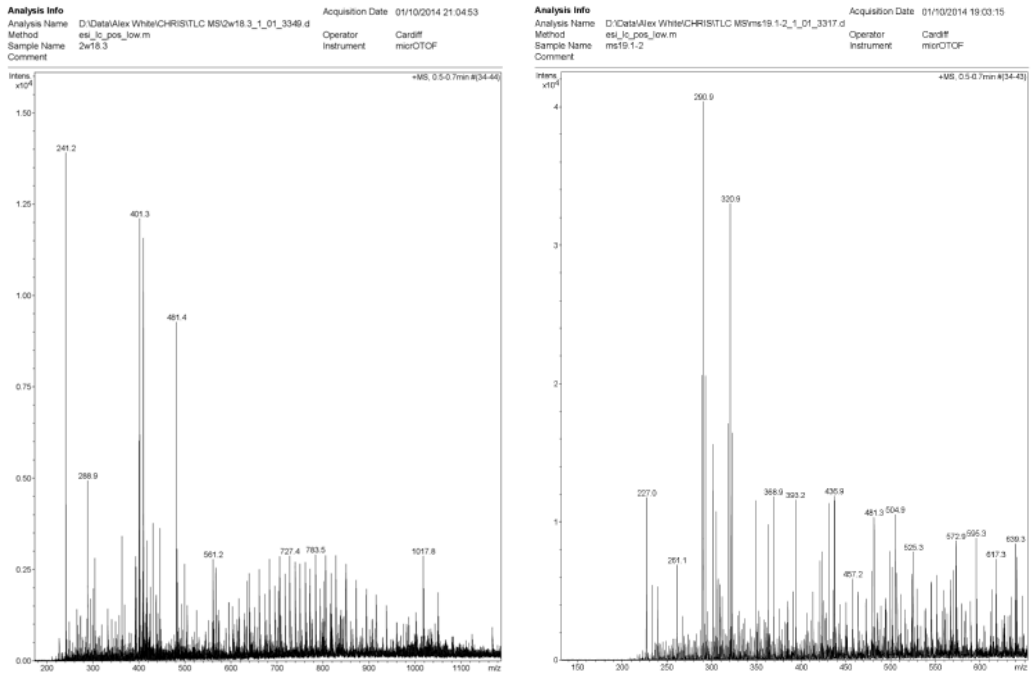


Figure 5-23 Mass spectrometry analysis of (A) Fraction 2w18.3 (*H. panicea*). (B) Fraction Ms19.1 9 (*A. oroides*)

The mass observed at *m/z* 282.3 with predicted mass of 281.3 $(M+H)^+$ found in both As10 and As10.1 fractions most likely represents 5-cis-C12-HSL (Figure 5-24), which has never been extracted from *C. crambe* but has been found to be produced by bacteria found on sponges of the same taxonomy class (Krick *et al*. 2007). This suggests that different sponge species may share common biosynthetic bacteria. The fact that this compound was identified both before and after separation with HPCCC indicated that HPCCC is a non-destructive method of separation and validated its use in purifying compounds from crude extracts.

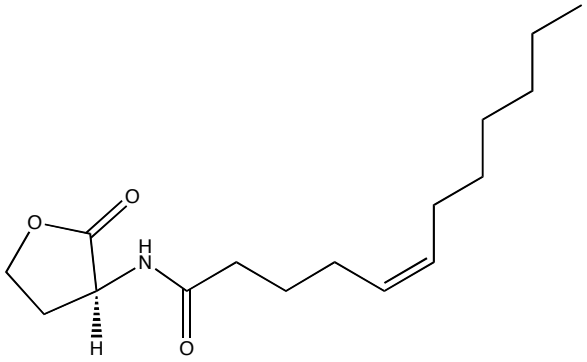


Figure 5-24 Chemical structure of (S,Z)-N-(2-oxotetrahydrofuran-3-yl)dodec-5-enamide. (Formula: $C_{16}H_{27}NO_3$; Mass: 281.3905).

The dibrominated peaks observed in fraction Ms19.1 (Figure 5-23) were also of interest with the monoisotopic peak of the lowest mass appearing at *m/z* of 288.9, therefore suggesting a mass of 287.9 $(M + H)^+$. A literature search using *MarinLit* found no dibrominated matches for this mass. The same applied to the other peak present in the mass spectrum of *m/z* 318.9 suggesting a mass of 317.9 $(M + H)^+$. As these compounds were extracted from *Agelas oroides*, simple fragmentation and rearrangement was attempted using the software ChemDraw Ultra 12.0, which produced one candidate that fitted the criteria for MW 287.9 and a possible analogue of the mass MW 317.9 (Figure 5-25). Other options may include six or seven membered rings linked directly to the bromopyrrole group.

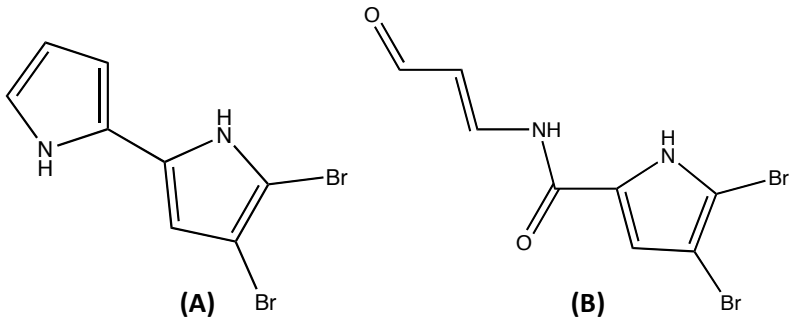


Figure 5-25 Chemical structure of (A) 4,5-dibromo-1H,1'H-2,2'-bipyrrole; Formula: $C_8H_6Br_2N_2$; Mass: 287.89 and (B) (E)-4,5-dibromo-N-(3-oxoprop-1-en-1-yl)-1H-pyrrole-2-carboxamide; Formula: $C_8H_6Br_2N_2O_2$; Mass: 319.88.

The structures suggested in Figure 5-25 have not been previously reported as marine natural products in *MarinLit* or *ChemSpider*. Fourteen non-marine natural product matches were found for isomers of formula $C_9H_8Br_2N_2O$ but none contained the dibromopyrrole ring characteristic of natural products produced by *Agelas oroides*. No matches were found in *MarinLit* for the suspected molecular formula and similar results were found for formula

$C_8H_6Br_2N_2$. As both these compounds are drug like (MW less than 550) and novel to marine organisms, they are worth further investigation.

In summary, 16 novel predicted masses were observed and 9 were potentially novel. When combining the novel and potentially novel data, six parent masses showed no matches for their expected ions: Three parent masses from As10.1 (*C. crambe*), and one from As10.3 (*C. crambe*), Ms19.1 (*A. oroides*) and 2w18.1 (*H. panicea*). However three of the active masses from *C. crambe* fractions were most likely the same displaying a similar *m/z* of 240.3. The most promising of all these leads was the mass observed in *H. panicea m/z* 293.5 as all expected ions were considered novel. In summary, this approach to dereplication is a good method of narrowing target leads and managed to reduce lead numbers by 85% (all previous TLC-MS results included). However, more detailed information was needed to make a more accurate comparison against a database. This could be completed by using higher energy MS methods to obtain accurate masses and fragmentation of the parent molecules.

5.4.3 HPLC - MS/MS and a molecular network as a dereplication strategy and lead compound identification

Several marine sponge samples were selected based on the quantity of material available and interesting collection information, such as varying sponge species, locations and environmental conditions upon collection. HPLC MS/MS was completed on these samples and the multiple standards selected (Table 5-3). Samples were grouped as follows: Welsh sponge extracts, Greek sponge extracts, HPCCC isolates and standards. These groups were then directly compared using a molecular network (section 5.3.6.5), to identify any structurally similar products found in the samples to the standards (Figure 5-26). All sample mass spectra were viewed on GNPS (GNPS 2015) and networks were visualised using appropriate: software Cytoscape 3.1.0 (Saito *et al*. 2012).

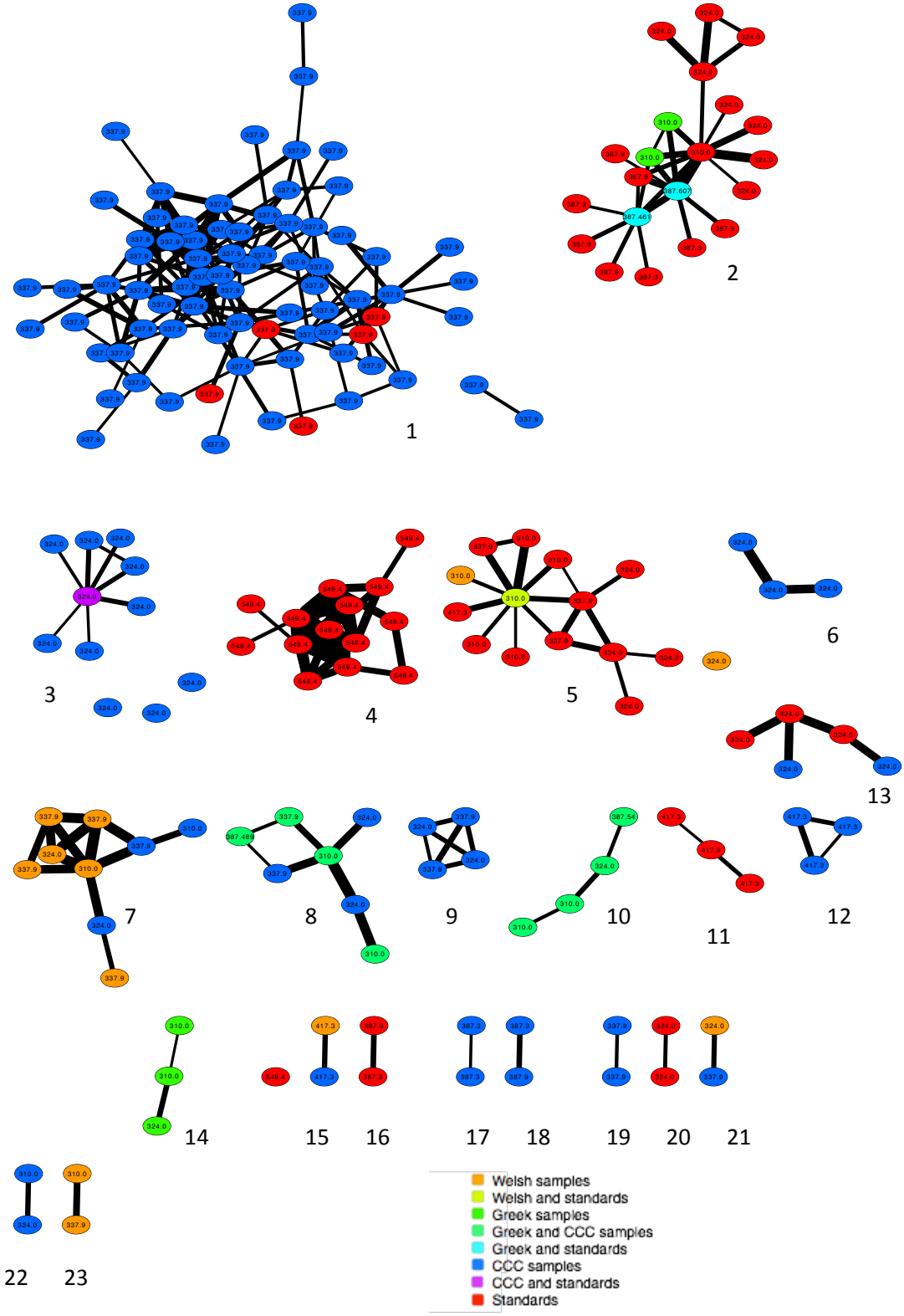


Figure 5-26 Molecular network of HPLC-MS/MS data. Each node represents a parent mass with an individual fragmentation pattern. The node may represent fragmentation patterns from more than one sample (if deemed the same). Each edge represents the cosine similarity between each node. The thicker the edge, the more closely related the compounds within the nodes are.

Following analysis of the network (Figure 5-26), any direct matches between the standards and the samples were considered identification of a standard within that sample. Any circumstance where a standard was directly linked within a network to sample lead to a postulation that structurally similar compounds were found in that sample to the standard. The clusters 1, 2, 3, 5 and 13 contained a mix of standards and samples and are discussed further in subsequent sections.

5.4.3.1 Analysis of the molecular network

5.4.3.1.1 Cluster 1

Cluster 1 (Figure 5-27) showed no direct overlap between spectra from the cholesterol standard (MW 386.35) and any extract tested. It did however indicate that some CCC fractions may be related to cholesterol due to the appearance of a spectra from the cholesterol standard and the tightly interlocking network. The parent mass observed was not that of cholesterol, with an *m/z* 338.0 and was peak picked in (MS1) as it represents the mass of another standard used in this experiment. However analysis of the spectra shown in Figure 5-28 appears to represent a sterol like product and shows a small parent mass peak of cholesterol. This was confirmed when the spectra were compared (Figure 5-28).

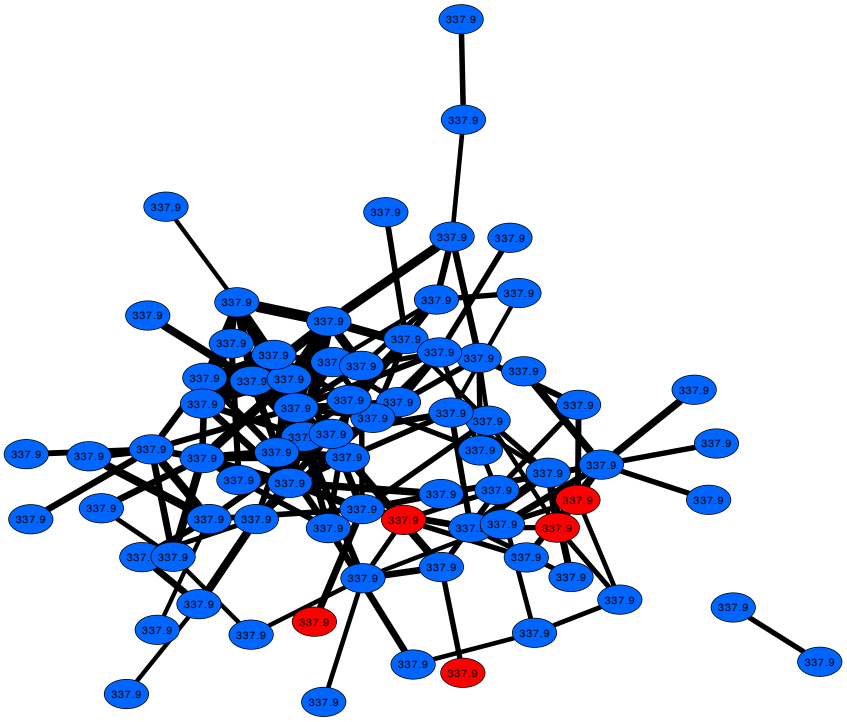


Figure 5-27 Cluster 1 red nodes represent standards and blue nodes represent samples from CCC separation.

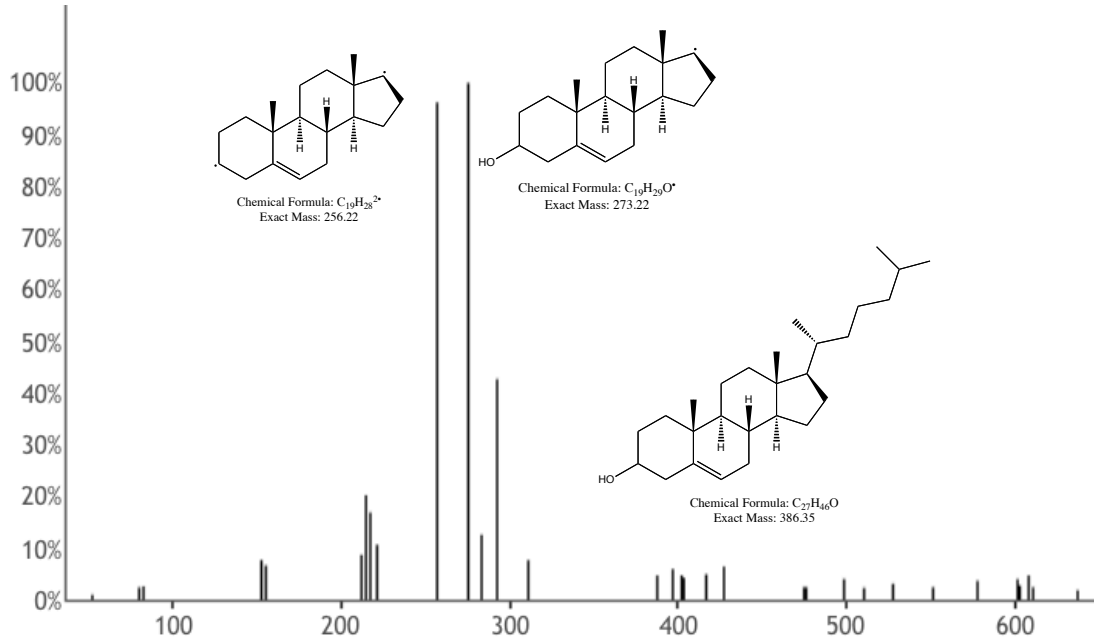


Figure 5-28 Comparison of MS/MS fragmentation pattern of Cholesterol standard and fraction 2w18.3 showing similarities between the compounds and suggested fragmentation.

Analysis of the potential fragmentation patterns of cholesterol (Figure 5-28**)** show how the principal fragmentation peaks, 256.97 and 274.98, observed in both the cholesterol standard and fraction 2w18.3, may have been formed. However, the intensity of the peaks observed and some of the other peaks were in different ratios. Results from the network suggest that the compounds detected were not the same but it was likely that fraction 2w18.3 contained a sterol but of slightly different structure.

Upon reviewing the workflow, there is a possibility that cholesterol was trapped in the column because it is a non-polar compound and therefore may have greater affinity for the structure of the stationary phase. It was also noted that cholesterol-like molecules only appeared in other samples post injection of the standard and therefore its appearance was most likely a contamination.

5.4.3.1.2 Cluster 2

The 387.461 blue node seen in Figure 5-29 contained spectra from the standard oroidin and the extract Ms19 (*Agelas oroides*) (Figure 5-30).

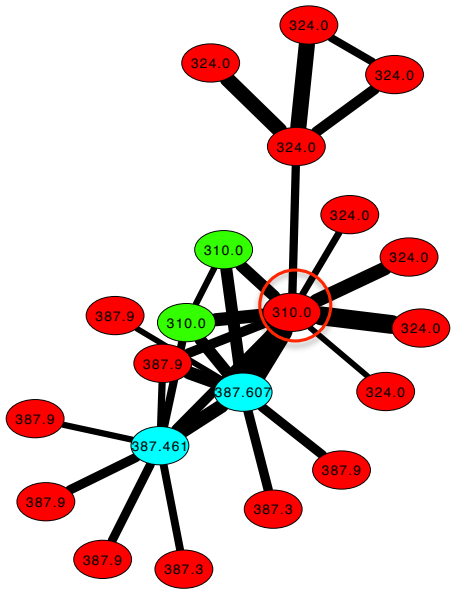


Figure 5-29 Cluster 2 with two matched nodes in blue (Greek sample and standard), two Greek samples in green and multiple sample nodes in red.

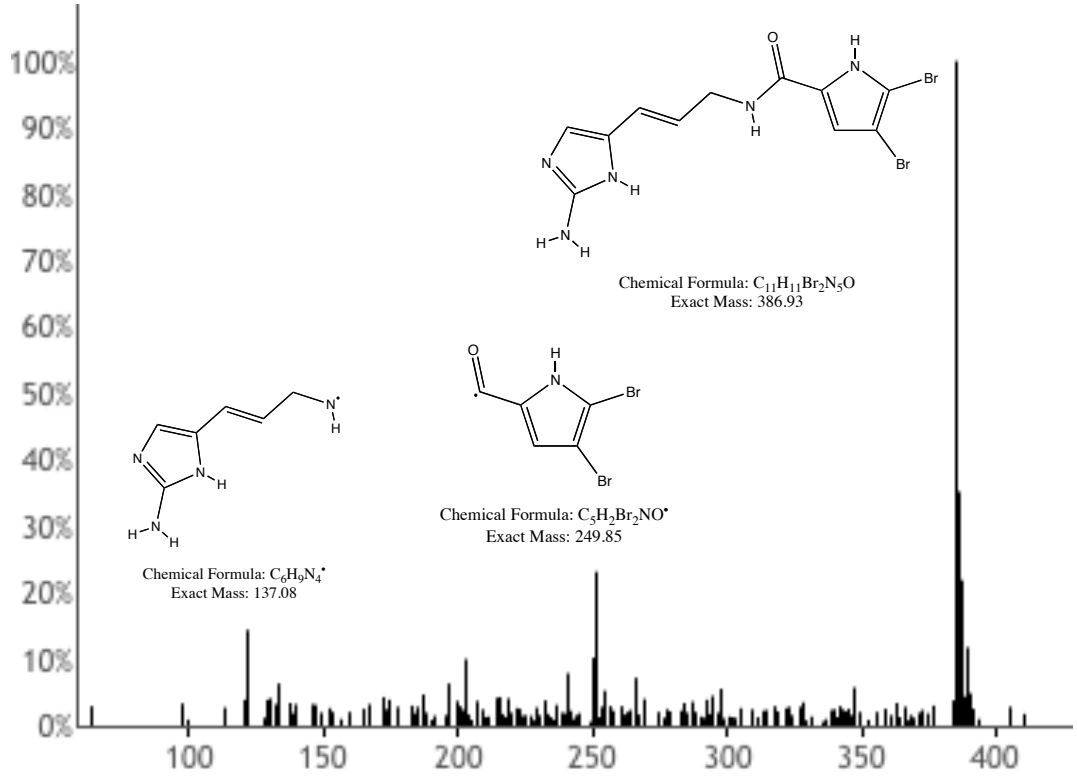


Figure 5-30 Comparison of MS/MS fragmentation pattern of Oroidin and extract Ms19 (*A. oroides*) and suggested fragmentation.

These samples displayed almost identical retention times of 649.06 s and 649.02 s respectively. Both also displayed peaks at *m/z* 251.85 and 139.09, which could be explained with the fragmentation of oroidin shown in Figure 5-30.

The 387.607 blue node seen in Figure 5-29 displayed an almost identical fragmentation pattern to 387.461 with the exact same masses of fragments identified (Figure 5-30) occurring again but with different intensities. The network indicated that they display a close similarity as demonstrated by the thickness of the edge between them. Both nodes also contain fragments found in oroidin and Ms19, further confirming that they were the same compound. Oroidin was also tentatively identified as a compound found in extract Ms19, using TLC-MS in section 5.4.1.3 thus validating both methods of identification and dereplication.

The green nodes also represent spectra from the Greek sample extract Ms19 but display a parent mass of *m/z* 310, they show similarities between themselves, the standard oroidin and the sample identified as oroidin. They also display a strong resemblance to the node circled in red, which the network identified as hymenidin showing major peaks at *m/z* 173.94, 139.10, 122.07 and 188.96 (Figure 5-31).

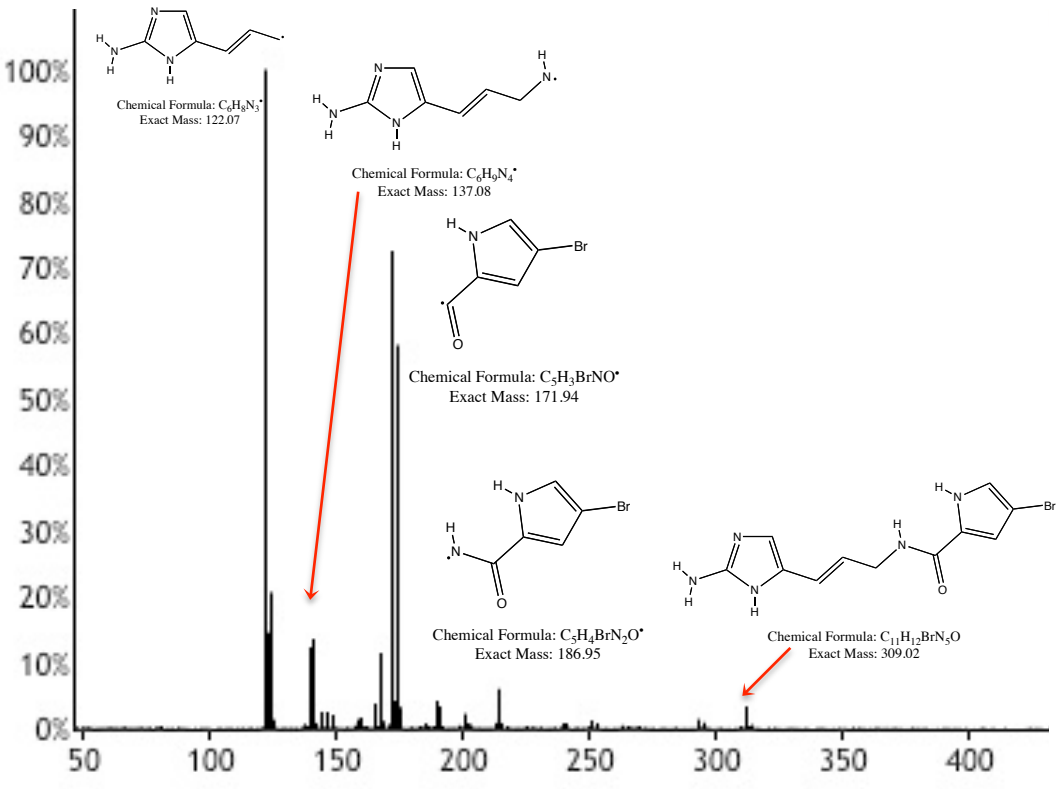


Figure 5-31 Comparison of MS/MS fragmentation pattern of hymenidin standard and extract Ms19 (*A. oroides*) and suggested fragmentation.

MS/MS spectrum of extract Ms19 shows clear fragments at *m/z* 173.94, 139.10 and 122.07 but no fragment at 188.96 (Figure 5-31), all fragments could be explained by the potential fragmentation of hymenidin (Figure 5-31). However, upon MS/MS spectrum analysis of the hymenidin standard (A, Figure 5-31), *m/z* 188.96 was of the lowest intensity of all the major peaks and upon closer visual analysis of the mass spectrum, a peak of relative low intensity was observed, which was not within the top 20 in this spectrum. Therefore, extract Ms19 most likely contained hymenidin if not a very similar compound. These two spectra displayed almost identical retention times of 168.39 s and 168.44 s respectively, suggesting that they were likely the same compound.

5.4.3.1.3 Cluster 3

Cluster 3 shows multiple HPCCC samples matched to cholesterol although again the parent mass of the nodes pictured do not match that of cholesterol (Figure 5-32). Unfortunately, upon closer visualisation of the individual spectra, the peaks were not very well resolved and there was a lot of background noise making interpretation of data impossible.

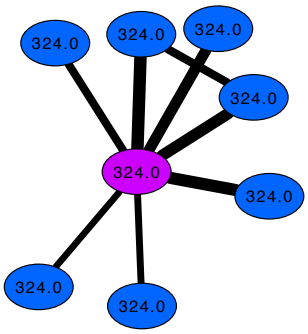


Figure 5-32 Cluster 3 with CCC samples matched to standard highlighted in purple.

5.4.3.1.4 Cluster 5

Extracts 2w345 (*Hymeniacidon perleve)*, 2w9 (*Amphilectus fucorum)* and the sceptrin standard have spectra matched in central yellow 310.0 node shown in Figure 5-33, with all surrounding red nodes also linked to the sceptrin standard. The orange 310.0 node represents extract 2w14 (*Halichondria panicea* in direct competition with *Hymeniacidon perleve)*, the edge between the yellow and orange node indicates structural similarities.

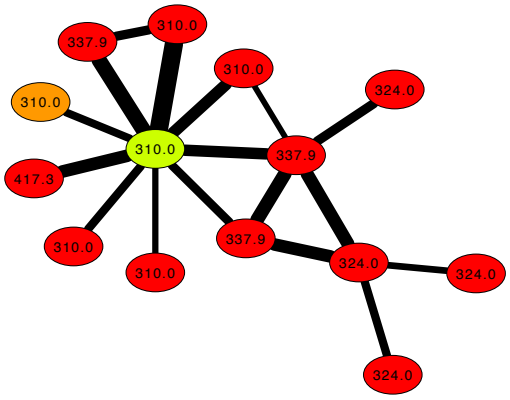


Figure 5-33 Cluster 5 with a Welsh sample node in yellow and a matched node in orange and multiple standard nodes in red.

From the MS/MS spectra (Figure 5-34), a very similar pattern appears to occur with the other Welsh sample extracts and the sceptrin standard with minor differences in the principal peak intensities. The retention times were also very similar at 96.31 s, 96.36 s, 96.311 s and 96.321 s for extract 2w14, which indicate that they most likely represent similar or the same compounds. The principal peak was identified at *m/z* 310, which interestingly is not the mass of sceptrin but was recognised by the network for its similarity to hymenidin.

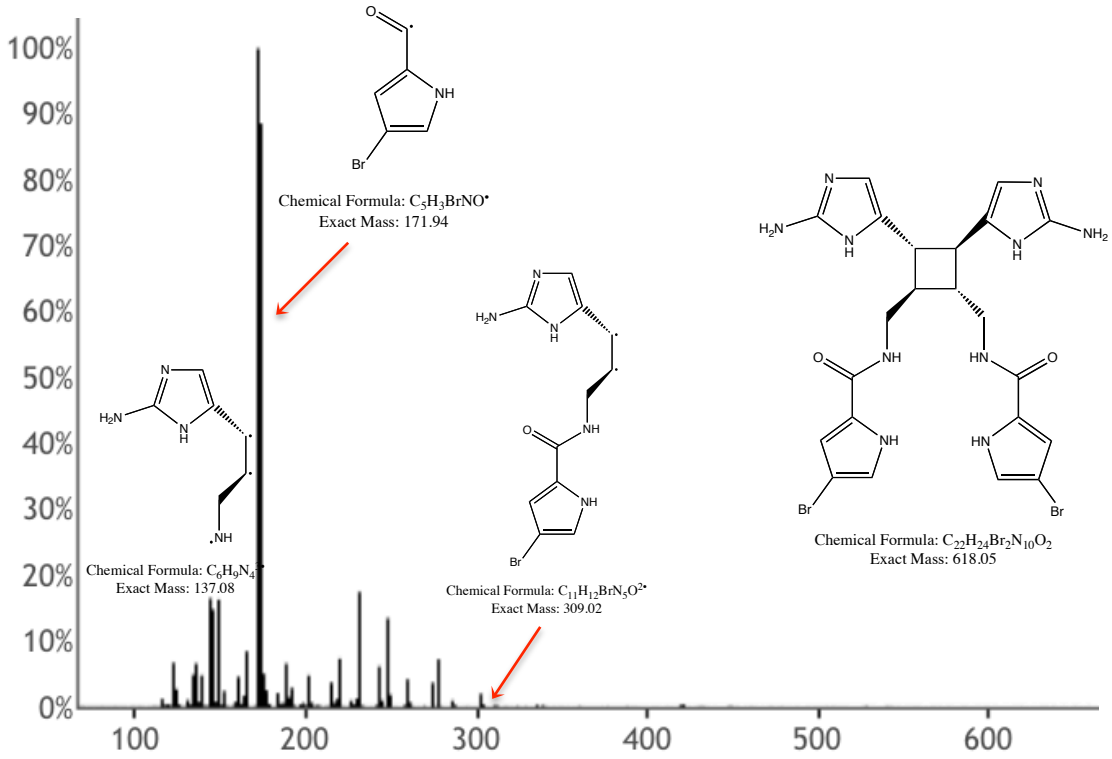


Figure 5-34 MS/MS fragmentation spectrum of extract 2w345, extract 2w9, sceptrin standard and extract 2w14 and suggested fragmentation.

However, the expected fragmentation of sceptrin fits the data as cleavage of the cyclobutane ring produces two fragments, both of mass *m/z* 309.02, which are essentially two hymenidin molecules (Figure 5-34). Fragmentation of the weakest bond of sceptrin produces molecules of mass *m/z* 171.94 and 137.08, which are observed as the principal peaks in the spectra of all the molecules found within this cluster (Figure 5-34). Identification of this compound shows that brominated compounds were present in Welsh samples, none of which were identified in the activity based TLC-MS analysis. It also revealed an interesting mix across species, with sceptrin appearing only in the *Halichondria panicea* species in a competitive environment.

5.4.3.1.5 Cluster 13

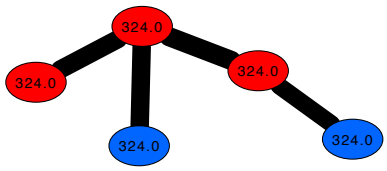


Figure 5-35 Cluster 13 with two CCC nodes and three standard nodes.

Each node within Cluster 13 displays the parent mass *m/z* of 324.0 (Figure 5-35), which in theory represent the parent mass of 10Z-hymenialdisine. The standards represented in this cluster are cholesterol, manoalide and oroidin. All nodes within this cluster also displayed a distinct individual peak at *m/z* 186.23, none of which showed any structural similarity to the potential fragmentations of the standards. This peak appeared in multiple samples at exactly the same retention time of 773 s, and is most likely a contaminant in the system.

5.4.3.2 Summary of the use of a molecular network as a dereplication strategy

The molecular network produced for this HPLC-MS/MS data proved extremely effective at identifying standards or similar compounds within extracts but some limitations were clear. Firstly, the method employed only matched the parent mass of the standards. This helped to increase sensitivity when attempting to identify standards but did not identify novel parent masses. Also all parent masses of standards were searched for each individual standard run, this clouded the parents masses identified for some standards *e.g.* Cholesterol (cluster 1) and Sceptrin (cluster 5). Some of the external clusters that are not attached to standards indicated the presence of some interesting compounds but were not worth exploring further as the possible parent mass was not identified. There was no indication of activity and there was no link to a standard to help determine the structure. In summary, the method employed proved useful in dereplication and prevention of rediscovery, but not for identifying potential novel compounds. The completion of the network using this HPLC-MS/MS technique and the subsequent identification of parent masses within samples from the standards validated its use and could therefore be utilised to mine the active parent masses identified from TLC-MS. A more complete network could also be formed by the introduction of more standards, potentially linking similar clusters together.

The problem of not identifying further leads could be addressed by continual scanning rather than peak picking during the gradient run. This would undoubtedly identify a larger number of parent masses and therefore more nodes in the network. The disadvantage of this is as fragmentation is occurring during separation, the parent peak will represent the largest peak, which could actually be a fragment of a parent mass. A further disadvantage of this is it is not activity based even though it may identify a novel compound by chance. This could be completed by identifying an analogue to a known active compound and observing a simple difference in parent mass and therefore its structure, essentially using nature as a method of producing analogues. This however would take in depth analysis of the network but is achievable.

5.4.4 Assessing the effect of environmental competition on the production of secondary metabolites

Samples of *Aplysina aerophoba* were collected from Samos (section 4.1.1). The specimens were collected via snorkelling at a depth of between 5 to 8 m. The first sample (C) was collected from a competitive environment, where the specimen was surrounded by two other species: *Crambe crambe* and *Chondrosia reniformis* (Figure 5-36). The second sample (I) was found in an isolated environment with no spatial competitors around (Figure 5-36). One interesting note, upon collection, was that one of the competitive sponges *C. crambe* is known to have high bioactivity and have a negative association with its neighbours through secondary metabolite production (Turon *et al*. 1996; Becerro *et al*. 1994).

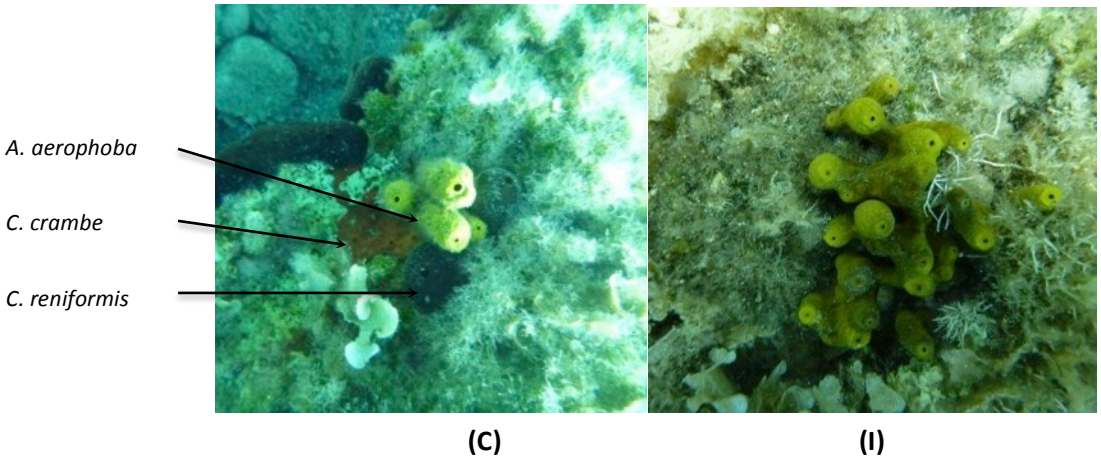


Figure 5-36 Digital underwater photographs of (C) *A. aerophoba* (yellow tubular sponge) in a competitive environment, surrounded by other sponge species (*C. Crambe* and *C. reniformis*) and (I) *A. aerophoba* in an isolated environment.

5.4.4.1 Extraction of crude extracts

An isolated sponge (2.545g) and competitive sponge (2.500g) were ground to a fine powder (section 2.2.2) and then extracted using three solvents of increasing polarity (section 2.2.3). This resulted in three fractions for each sponge: 1I, 2I and 3I and 1C, 2C and 3C; with the preceding numbers increasing with polarity. The crude extracts were then further separated using TLC (Table 5-14).

Table 5-14 TLC summary of crude extracts (hexane, acetone and methanol) of isolated (I) and competitive (C) *A. aerophoba*. TLC plates were eluted using the following solvent systems: hexane extracts[1]; 3:7 (acetone : hexane); acetone extracts[2], 1 : 9 (methanol : DCM); methanol extracts[3], 16 : 2 : 1 : 1 (acetone : methanol : water : acetic acid). Fractions highlighted in red were only found in the 'competitive' sample.

Extract	Appearance	Rf
1I[1], 1C[1]	Orange spot	0
	Yellow spot	0.25
	Green spot	0.35
	Green spot	0.4
	Green spot	0.45
	Yellow spot	1.0
2I[2]	UV_{254} active	0.8 (2I1)
2C[2]	UV_{254} active	0.8 (2C1)
	UV_{254} active	0.7 (2C2)
	UV_{254} active	0.6 (2C3)
3I[3], 3C[3]	UV_{254} active	1,2,3,3.5, 5,6.5,7,9

5.4.4.2 Separation of compounds only found in a competitive environment using thin-layer chromatography

Only one separated component was observable under UV light in the isolated sponge sample but three components were visible in the competitive sponge sample when comparing the crude acetone extracts of both samples. Therefore, the acetone extract was chosen for further investigation. The crude acetone extract of the competitive sponge (2C) was separated using preparative TLC (2:8 acetone : hexane) into three fractions 2C1, 2C2 and 2C3 (Figure 5-37).

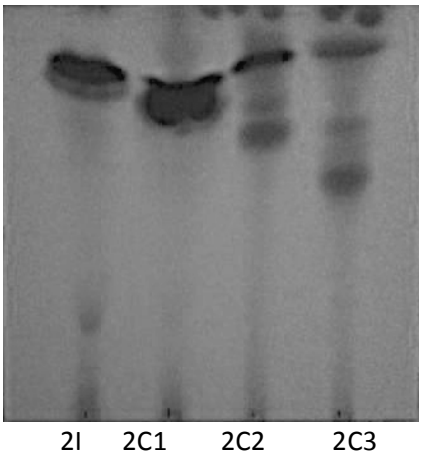


Figure 5-37 TLC comparison (6: 4 acetone : hexane) of fractions of the acetone extracts from *A. aerophoba* collected in isolated (2I) and competitive environment (2C1, 2C2 and 2C3). Different compounds were clearly present in fraction 2C2 and 2C3 and not in the isolated sponge extract 2I.

5.4.4.3 Identification of the fractions separated from the acetone extract of the competitive sponge (2C)

The ^{1}H NMR data of 2C1 (Table 5-15, Figure 5-38) that the compound detected could potentially be drug-like. The compound consisted of between 26 to 29 hydrogen atoms, implying that it most likely contained less than 20 carbon atoms and therefore was of low MW. It was also likely to be lipophilic as it contained multiple aromatic groups and double bonds. This NMR did not provide a conclusive picture of the structure of this compound by itself and more data was needed to fully identify the compound. ^{13}C NMR was also performed in an attempt to provide a clearer picture but the results were inconclusive due to the relatively small quantity of sample.

Table 5-15 Analysis of ^{1}H NMR (500 MHz) data for extract fraction 2C1 (competitive *A. aerophoba*) in DMSO-d6.

δH	Integral	Interpretation
8.40, t	1H	Appear in aromatic region, therefore could be assigned as protons on benzene ring
8.35, t	1H	
7.59, s	2H	
6.59, d	2H	Doublet and double doublet. Appear between the region of aromatic and protons attached directly to C=C double bond. Double doublets arise when a proton couples to two chemically different protons. This can occur in both benzene ring and double bonds.
6.36, dd	2H	
5.72, d	1H	Appear in region of a Proton attached directly to a double bond
5.26, d	1H	
4.69, q	1H	All integrate to one proton. Appear between the region of C=C and CH_3, CH_2 and CH groups attached to electronegative atoms or groups such as O, N, F, Cl, CN, C=C and C=O.
4.06-4.10, m	1H	
3.91-3.95, m	3H	Appear as aliphatic protons
3.83-3.86, m	1H	
3.66, s	5H or 6H	Possibly an aliphatic ring structure
3.61-3.65, m	1H	Aliphatic protons
3.48-3.53, m	1H	
3.17-3.25, m	1H or 2H	
1.23-1.37, m	2H or 3H	Likely alkyl protons and possibly correspond to CH_2 or CH_3 group that is not attached to any adjacent electronegative groups

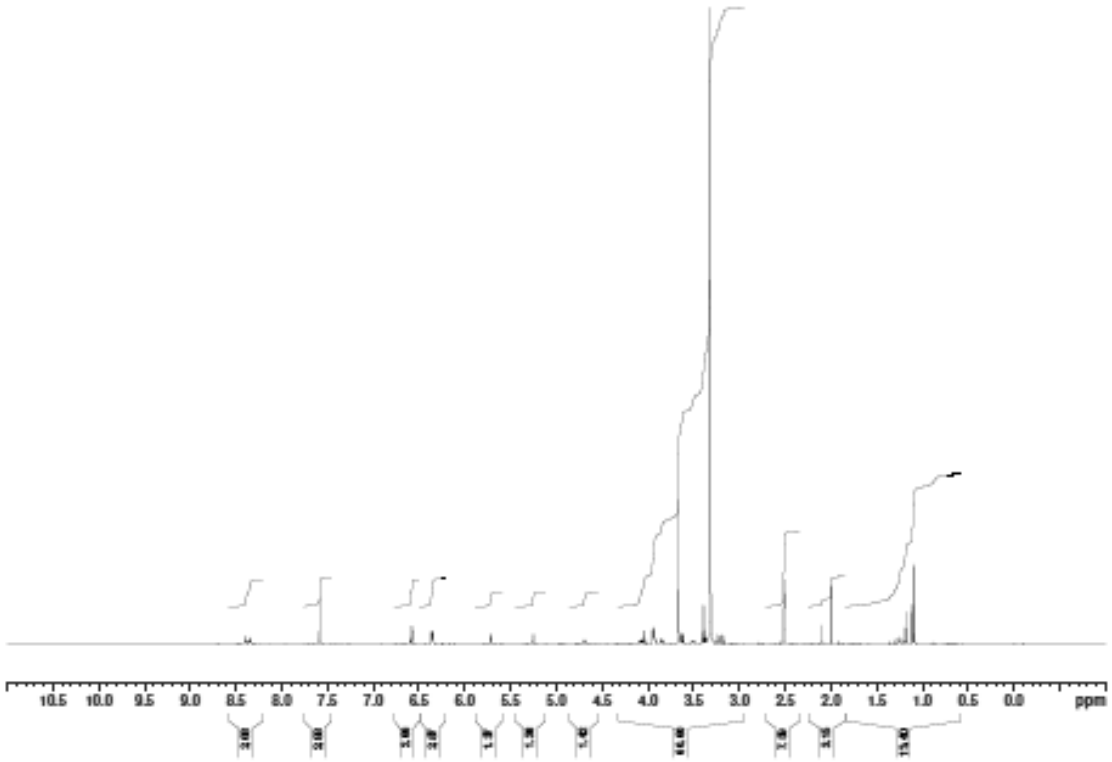


Figure 5-38 ^{1}H NMR (500 MHz) data for extract fraction 2C1 (competitive *A. aerophoba*) in DMSO-d6.

The high resolution MS data (Table 5-16 and Figure 5-39) for compound 2C1 predicted a mass of 662.4519 $(M + H)^+$, which did not match the interpretation of the NMR data. This may be due to symmetry in the molecule or presence of a mixture of compounds in the NMR sample. The data also showed that extract 2C1 did not contain the halogens Cl or Br as the characteristic 1 : 1 and 3: 1 peaks did not appear. The formulas suggested were $C_{30}H_{15}N_7O_9$ or $C_{28}H_{58}N_{10}O_{8,}$ which did not match any known compounds found in *A. aerophoba* indicating that this may be a novel compound.

Table 5-16 Predicted mass summary from MS spectra of extracts 2C1, 2C2 and 2C3 (*A. aerophoba*).

Compound	Predicted mass $(M + H)^+$
2C1	662.4519 (accurate MS)
2C2	662.3
2C3	853.5

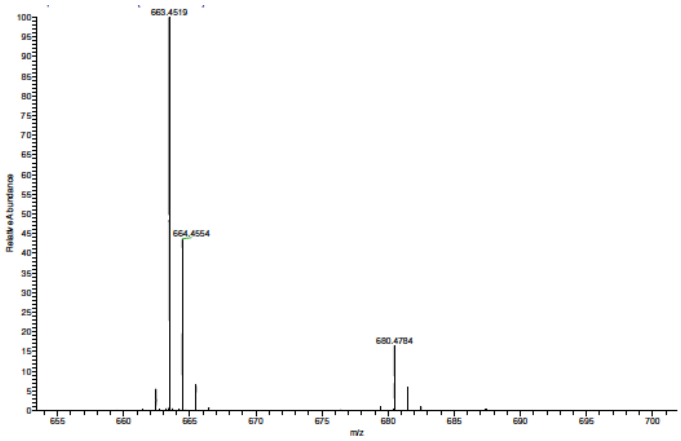


Figure 5-39 High resolution MS of extract 2C1 (*A. aerophoba*) with principal peak of m/z 663.4519.

Extracts 2C2 and 2C3 contained two compounds, which were not found in the isolated sponge sample, with the molecular weights of 662.3 and 853.5, respectively. Similar to 2C1, their molecular weights were compared to those of known compounds from *A. aerophoba* and again no matches occurred, suggesting these were also unknown metabolites from this sponge. The masses were also compared to those of common plasticisers and no match was found. Unfortunately, neither of these compounds showed antibacterial activity and the masses of all three compounds identified were above the molecular weight limit of 550 set for this project. They were not considered drug-like and were not investigated further.

Compounds from extracts 2I1 and 2C1 were the only common components isolated from the two samples, as determined by TLC. The percentage weight by weight recovered for compounds 2I1 and 2C1 were 4.3% *w/w* and 6.8% *w/w*, respectively. This suggests that the

competitive sponge produced higher concentration of the same compound compared to the isolated sponge. Further studies would have to be completed to confirm that spatial issue was the only factor that caused this difference. Alternatively, it may have been caused by the different sizes of the sponges or the fact that different parts of the sponge were sampled. Although sponges may have hot spots of metabolites (Puyana *et al*. 2003), the entire collected samples were all efficiently homogenised. Therefore, an even mix of compounds was expected. Some studies have previously used core tissue and radial wedge tissue in an attempt to represent the sponge more equally (Puyana *et al*. 2003) however investigating this further was not a principal aim of this study.

Although not considered drug-like by the stipulations set out in this study, compounds 2C2 and 2C3 have provided useful information. Both compounds were extracted from the competitive sponge sample and neither was extracted from the isolated sponge sample. Therefore, *A. aerophoba* could have produced these compounds as a form of chemical defence in response to spatial competition. There are limitations to this assumption as many other factors may affect the secondary metabolite production within a sponge, such as size, biotransformation upon tissue damage and heterogeneous distribution of compounds throughout sponge tissue (Puyana *et al*. 2003). It has been speculated that competitive interactions may be dependent upon sponge size with larger sponges producing more toxic bioactive metabolites (Luter and Duckworth 2010). Figure 5-36 shows that the sponge in isolation was actually larger than the sponge in competitive environment; therefore the suggestion that larger sponges are more toxic may not be true for this species. Although some studies have reported that surrounding competition is not likely a major contributing factor to compound variation (Luter and Duckworth 2010), these studies were not carried out on *A. aerophoba* and hence this may not be true for this sponge species.

A similar experiment was performed on two samples of *Halichondria panicea*, also found in these two different environments. A difference was also observed in metabolite production (Figure 5-40), but no antibacterial activity of these ‘extra’ compounds was observed, therefore they were not explored further.

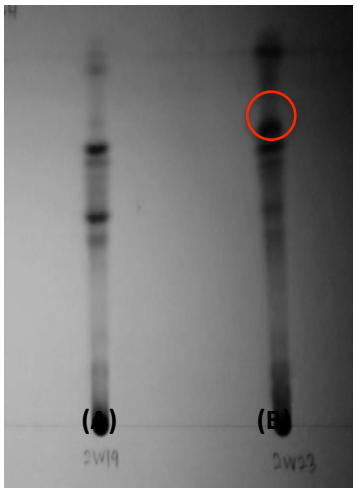


Figure 5-40 TLC comparison (6: 4 acetone : hexane) between acetone extracts of *Halichondria panicea* collected in isolated environment (A) and competitive environment (B) with an extra metabolite found in the competitive species highlighted in red circle.

5.5 Conclusion

The principal objectives of this chapter were the identification of novel active parent masses and dereplication of active molecules found within the marine sponge samples. Multiple known natural products have been identified using the various methods of isolation and identification, which proved that the methods used were effective at separating active compounds from inactive material and identifying them. The methods employed also succeeded in isolating and dereplicating some unknown parent masses, which are potentially novel and therefore exciting leads for continuation of work beyond this project.

Bioautographic overlay TLC proved a useful method of identifying differing areas of activity within a crude sample but provided little information about the compounds responsible. For the first time, marine sponge extracts have been analysed by direct bioautographic TLC, followed by TLC-MS from a duplicate plate, allowing rapid dereplication of parent masses from confirmed antibacterial fractions. Parent masses and other limiting information such as the sponge species and isotope peak splitting provided instant dereplication of known molecules. This led to the detection of 21 potential lead compounds, of unknown structure, that require further research. The information gained using this process was however still limited as potentially unknown products may have matched known molecular weights from marine natural products. This does not condemn its use as a lead identifier, but it has to be assumed it is not all encompassing.

Activity guided template solvent systems were developed for HPCCC allowing the separation of active compounds from crude extracts with little or no loss of material. The isolation of differing parent mass molecules have shown that Greek and temperate Welsh sponges are rich sources of diverse active metabolites and that the template solvent systems developed for each extract were excellent starting points in the isolation of these active compounds from marine sponges. The record of retention time of each active fraction, the ease of adjustment of a four solvent based system and the direct scalability of HPCCC (DeAmicis *et al*. 2011) make this method perfect for future separations and targeting of novel active molecules that require full characterisation.

Direct comparison to standards was also completed using HPLC-MS/MS to separate parent masses from a crude mixture and then fragment parent structures to generate extra information about the structure of a molecule. These fragmentation patterns were then

directly compared to one another using a computer generated molecular network, which analysed the cosine similarity between the fragmentation patterns of each parent mass. This method is currently rapidly developing for use in the analysis of bacteria, including those found in sponges (M. C. Wilson *et al*. 2014) but has not been used to analyse sponge extracts directly. This study completed HPLC-MS/MS searching for the parent masses of standards to ensure no compounds were missed. The disadvantage of this method is it does not actively search for masses of novel compounds. Analysis of the molecular network and the spectrum that created it showed that multiple standards were present within the samples analysed.

A simple experiment on the effect of environmental competition on the secondary metabolite production in marine sponges showed species found in a competitive environment produce more metabolites than those in isolated environments. As discussed in section 5.4.4.3, there are various limits to this assumption, including the small scale of the study, the difference in sizes or sponges species and the unknown information about sponge growth and environment prior to collection. No definitive answers were ascertained in this study due to these natural circumstances that cannot be controlled however it has at least shown that targeted collections are worthwhile. This encouraging initial result will be investigated further in future extractions and in chapter 6 with the effect of environmental competition on microbial populations.

Chapter 6
Bacterial cultivation and isolation of antibacterial compounds from Welsh sponge associated bacteria

6 Bacterial cultivation and isolation of antibacterial compounds from Welsh sponge associated bacteria

6.1 Chapter introduction

6.1.1 The role of sponges as hosts for symbiotic bacteria

Marine sponges are fertile hosts of a diverse microbial community found throughout the sponge organism both intra- and extracellularly (Lee *et al*. 2001). A sponge and its microbial community form a very close relationship, which is thought to be truly mutualistic in some species, providing a beneficial environment for both. The sponge provides the bacteria with protective shelter through internal and external surfaces that are nutrient rich and therefore full of food compared to the vast ocean. The bacteria found in or on a sponge are thought to aid the sponge's digestion of nutrients through translocation of metabolites by nitrogen fixation (Wilkinson and Fay 1979; Freeman and Thacker 2011), nitrification and photosynthesis (Lee *et al*. 2001). The bacteria may also provide additional stability to the skeletal structure of the sponge (Wilkinson *et al*. 1981) and participate in the sponge's chemical defence against predators such as nudibranchs (Proksch 1994). Sponges are such fertile hosts of bacteria that microbial concentrations can exceed that of seawater by two orders of magnitude (Friedrich *et al*. 1999).

Marine sponges have been identified in previous chapters as producers of potent antimicrobial compounds (section 4.4.6) and it would seem counter intuitive that they are good host for bacteria. Fascinatingly, in attempts to determine the root cause of the antibacterial activity of sponge extracts, it was found that they often show activity against terrestrial pathogens as seen in section 4.4.6 but rarely against marine bacteria (Amade *et al*. 1982; Amade *et al*. 1987; Newbold *et al*. 1999). This suggests the production of these compounds by the sponge or bacteria is not inhibitory to potentially co-occurring or harmful bacteria. While the background evidence identified indicates that this symbiosis can be a truly mutualistic symbiotic relationship, this has been difficult to prove in the laboratory. It is possible that some sponge bacteria are parasitic and even damaging to the sponge host. This study will refer to any bacteria isolated from a marine sponge as associated and not symbiotic and in no way indicates true mutualism.

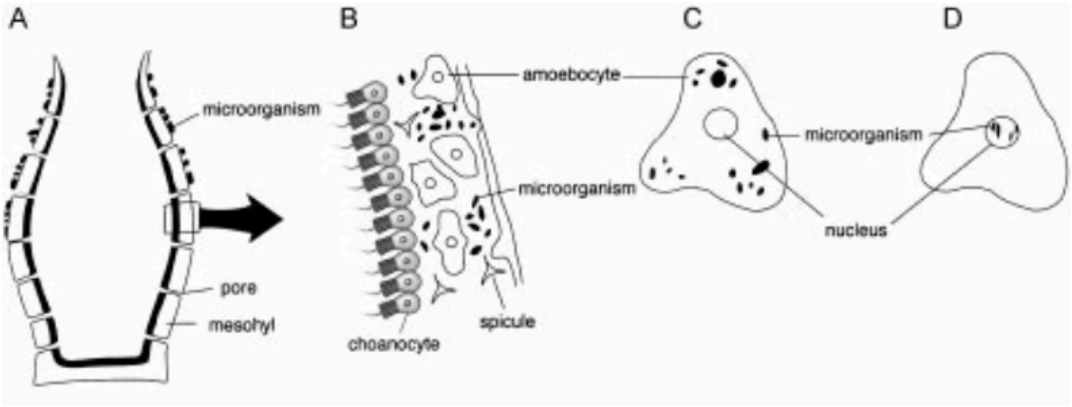


Figure 6-1 Schematic diagram of the symbiotic relationship between sponges and microorganisms. A, extracellular exosymbiosis. B, extracellular endosymbiosis. C, intracellular symbiosis. D, intracellular symbiosis taken from Lee *et al.* (2001)

6.1.2 The role of microbes and their sponge host as the source of secondary metabolites

Estimations suggest that up to 50% (Hentschel *et al*. 2012) of a sponges biomass is made up of bacteria, although the nature of the relationship between the sponge and its symbiotic bacteria is not always obvious. It has been speculated that due to the molecular similarity of the chemicals isolated from both microbes and bacteria, many of the products thought to originate from sponges are actually of microbial origin (Faulkner 1998). It is possible that the sponges produce these by themselves, the bacteria use secondary metabolites from the sponges and chemically alter them, or the bacteria alone produce these chemicals (Lee *et al*. 2001). Bacteria collected from sponge samples have been found to produce interesting secondary metabolites without a sponge association. Multiple compounds originally attributed directly to a sponge for their manufacture are now recognised to be produced by associated bacteria and have been grown in the absence of the host sponge (Lee *et al*. 2001). An example of this is the brominated biphenyl ethers formerly attributed to the sponge *Dysidea. Sp*, which are now known to be produced by an associated V*ibrio sp*. bacteria and not the sponge host (Elyakov *et al*. 1991) (Figure 6-2). Another interesting example is the discovery of the phylum *Entotheonella*, which was found to be the producer of almost all the polyketides and peptides previously associated with its parent sponge host *Theonella swinhoei* (M. C. Wilson *et al.* 2014). A further relevant paper is that by Hu, Hamann, Hill and Kelly which investigated the concept that the manzamine alkaloids where generated by sponge associated bacteria rather than the parent sponge (J.-F. Hu *et al*. 2003). They successfully identified manzamine alkaloids and derivatives from actinomycetes and developed a method of screening strains as manzamine-producers using 16S RNA analysis.

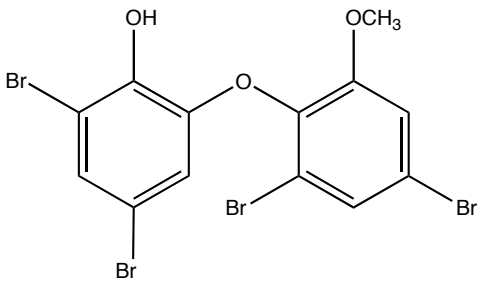


Figure 6-2 Chemical structure of 3,5-dibromo-6-(3,5-dibromo-6-methoxyphenoxy)phenol; formula: $C_{13}H_8Br_4O_3$; Mass: 527.72. Structure originally isolated from *Dysidea. sp*, now known to be produced by a associated bacterium and not the sponge host.

It is clear that is some situations the bacteria and not the sponge are true producers of the bioactive metabolites but this does not prove whether the bacteria require the sponge to produce them or whether a symbiotic relationship is present. Symbiosis is actually very difficult to truly prove. It is relatively simple to prove that bacteria need a parent sponge to proliferate by the inclusion of sponge material in agar, however this does not prove that this relationship is also beneficial to the sponge host (Hentschel *et al*. 2012). It may also be the case that true symbiotes may not grow in a laboratory due to the current scarcity of techniques to perfectly mimic the sponge host environment.

A method of identifying symbiosis may be to study growth specificity and one study of the microbiome of 32 sponge species found that some bacteria only colonise a small number of the sponge species studied (Schmitt *et al*. 2012). Conversely three species of bacteria where present in 70% of the sponges studied and it is thought that these probably arise through horizontal transmission through the surrounding sea-water. The sponge specific species however are more likely to be transferred via vertical transmission in sponge eggs and larvae, this method of transmission has been identified molecularly and microscopically (Schmitt *et al*. 2007). More striking evidence of symbiosis would be horizontal gene transfer between densely populated microbial communities and the host sponge and although some potential cases have been identified non are unequivocal (Hentschel *et al*. 2012).

6.1.3 Exploring sponge associated bacteria as producers of bioactive natural products

Only 1 to 5% of the microorganisms found on sponges are found to be cultivable (Amann *et al*. 1995) and the apparent presence of other microbes is only known through their genetic fingerprints. However, methods of culturing are improving with new low nutrient agars and broths as well as novel techniques of isolation, allowing isolation and exploitation of

previously uncultured microorganisms (Lam 2006). Not only are sponge microorganisms the potential major producers of the compounds responsible for activity, they are a particularly good route of exploitation for novel compounds as they are much easier to grow and harvest than marine sponges. Methods have vastly improved over the past 10 to 20 years, but limitations can be addressed using different approaches. The main disadvantage of the traditional method of directly culturing active microorganisms can result in down-regulation of the production of the bioactive metabolites (Laureti *et al*. 2011). This may be due to the lack of environmental stimulus and necessity to produce the metabolites for defence or protection. Another method of approach would be to identify the pathway responsible for the activity and express it a cultivable surrogate host (Jaspars and Challis 2014). This presents a relatively easy method of production post-isolation of compound compared to sponges where cultivation is difficult and may not reproduce the environment needed for the production of metabolites (Belarbi *et al*. 2003). Compounds could also be manufactured in the laboratory by synthesising or semi-synthesising any metabolite, which can be complex and expensive, but may improve activity (Zidar *et al*. 2014).

Interest in UK sponges has increased recently with samples being collected and exploited as a source of microorganisms, which show activity against a variety of different causative agents of infection (Margassery *et al*. 2012; Kennedy *et al*. 2009; Santos *et al*. 2010; Baker *et al*. 2009). This research has isolated multiple novel species of microorganisms but the majority have not identified the root cause of activity such as a novel compound. As recently as 2014, research in the UK has started to look at both chemical profiling of the sponge as well as bacteria characterisation (Viegelmann *et al*. 2014). This published study by Viegelmann *et al* (2014) however simply looked for common metabolite matches rather than common active metabolite matches.

Uniquely, the research in this thesis exploited clinically relevant activity found from microorganisms extracted from UK sponges as well as isolated and identified the compounds responsible for the activity. This project not only aimed to gauge the activity of microorganisms found in the unique environment around Wales (section 4.1.1), it also focused on identifying the pure culture responsible for activity using techniques such as 16S rRNA gene sequencing. These advanced techniques are progressively cheaper and more accessible, allowing complex sequencing of the complete spread of bacteria found on a sponge and identification of individual colonies responsible for activity. Activity identification can be achieved by growing bacteria extracted from the sponge samples on agar, before

isolating individual colonies and testing for activity. Active isolates can then be grown on a large scale enabling extraction of the compounds responsible for activity using the same extraction, isolation and activity testing methods employed for sponge material (chapter 4). Once extracted, direct comparisons between sponge samples and associated bacteria can be made through chemical profiling using the bioautographic overlay method combined with TLC-MS and molecular networking through HPLC-MS/MS (chapter 5).

6.2 Chapter aim and objectives

The primary aim of this chapter was to compare the antibacterial compounds extracted and isolated from sponge material to those isolated directly from sponge-associated bacteria. The objectives of this chapter were to:

- Cultivate unusual bacteria with potential antibacterial activity.
- Identify bacterial colonies that display antibacterial activity.
- Identify the bacterial species responsible for activity using rRNA analysis.
- Complete mass culture of antibacterial strains and extract compounds responsible for activity.
- Isolate novel active parent masses within the bacterial extract and dereplicate against standards and sponge extracts.

6.3 Materials and methods

6.3.1 Materials

6.3.1.1 Growth medium

- Sterile artificial seawater, Red Sea Coral Pro Salt (ASW) (Red Sea, UK).
    - Prepared as per manufacturer's instructions using deionized water.
- Tryptone soya agar (TSA) (Sigma-Aldrich, UK).
    - Prepared as per manufacturer's instructions.
- Lysogeny broth (LB) soft agar (LB soft agar) (Sigma-Aldrich, UK).
    - Prepared with 1 L of distilled water, 20 g LB broth, 5 g agar.
- Starch-Yeast-Peptone Sea Water (SYP) agar/broth.
    - Starch 10 g, yeast extract 4 g, peptone 2 g, artificial sea salt 33.3 g, +/- agar 15 g.
- Marine agar (MA).
    - Yeast extract 1 g, tryptone 5 g, artificial sea salt 33.3 g, agar 15 g.
- Seawater agar (SW).
    - Agar 18 g, artificial sea salt 33.3 g.
- Marine agar and marine broth 2216 (MA and MB) (Difco, BD, US).
- Anaerobic media testing.
    - 100 µL of homogenised sponge was pipetted into 200 mL of thioglycollate broth media (USP) (Oxoid, UK) and incubated at 25 °C for one month.
- Actinomycete isolation agar.
    - 2.2 % Actinomycete isolation agar (Sigma Aldrich, UK), 0.5 % glycerol, 3.33 % artificial sea salts.

6.3.2 Preliminary bacterial cultivation from Welsh marine sponge samples (*Cliona celata*)

6.3.2.1 Sample preparation

Samples of *Cliona celata (C. celata),* were collected *via* scuba prior to the commencement of this project, by Dr Julian Marchesi (Cardiff University) and suspended in a bacterial protectant (10 % v/v DMSO/collected seawater) and cryopreserved at −80 °C. The sample was defrosted for 16 h in a laminar flow cabinet. The following day, sponge pieces were

removed from the solution and any foreign particles were removed with sterile tweezers, the storage solution was kept as a control for testing. The sponge sample was rinsed with sterile artificial seawater. This was completed 4 times by re-suspending the sponge in sterile artificial seawater and shaking it for 5 min a shaking platform. After each rinse, the sponge was removed and re-suspended in fresh sterile seawater. The rinsed sponge was dissected into small pieces (approximately 5mm × 5mm) using a sterile scalpel, and homogenized using a sterile pestle and mortar. The homogenate was suspended in 20 mL of sterile artificial seawater. This suspension was serially diluted (1:10), three times.

6.3.2.2 Culturing of associated bacteria

The homogenised sponge, the turbid seawater it was found in, and (if collected) local seawater were all serially diluted three times to give a total of 12 different solutions to attempt to grow bacteria from. An aliquot of 100 µL of each solution was pipetted onto three different types of seawater-based agar (SW, SYP and MA unless described otherwise). The suspension was spread using a sterile spreader before being wrapped in parafilm, to prevent the agar drying out, and incubated at 25 °C. Each plate was checked regularly over two to three months looking for growth of individual colonies, which were removed and streaked on to nutrient rich marine agar (SYP) and the agar it had originally grown on. These plates were incubated at 25 °C for 2-3 days and single colonies were removed from the end of the streaks using sterile loops and used to inoculate a 1 mL solution of sterile 30 % v/v (glycerol / ASW). These samples were cryopreserved at −80 °C and kept as a record ready for identification. If the streaking process did not produce clear single colonies the process was repeated until single colonies were achieved.

6.3.2.3 Quantitative particulars of additions

6.3.2.3.1 Antibacterial (nalidixic acid) and antifungal (amphotericin) additives

- Amphotericin B (Sigma-Aldrich, UK). An aliquot of 30 µg mL^{-1} was added to all agar plates following the preliminary cultivation from *C. celata*. It was first suspended in DMSO 2.5 mg mL^{-1} due to poor solubility in water. The resulting solution was mixed thoroughly before being filtered to ensure sterility.
- Nalidixic acid (Sigma-Aldrich, UK). An aliquot of 25 µg mL^{-1} was added to agar by firstly mixing to a concentration of 0.005 mg mL^{-1} with sterile seawater and filtering the resulting solution before adding to the agar mix.

6.3.3 Deferred antagonism assays

An aliquot of 5 µL of pure culture was spotted onto an MA plate and incubated at 20 °C for 3 to 5 days until the culture had formed a colony of 0.5 cm to 1 cm in diameter. The agar plate was removed from the incubator and overlaid with 10 mL of LB soft agar, which had been inoculated with 50 µL of prepared culture (section 2.5.3) (E. *coli* (NCIMB 12210), MSSA (NCIMB 9518) or MRSA (NCTC 11939). The overlaid plates were incubated for 16 h at 28 °C. A zone of clearance visualised in the overlaid agar indicated the production of an antimicrobial compound by an isolate (Kennedy *et al*. 2009).

6.3.4 Bacterial cultivation from a variety of Welsh sponges collected in varying environments

6.3.4.1 Sample preparation

Samples from varying conditions were collected via SCUBA and wading (section 4.4.2) but upon arrival in the laboratory were treated differently for bacterial cultivation than they were for solvent extraction. As mentioned in section 4.4.2, a portion of the sample was removed using a sterile scalpel for bacterial cultivation by suspending in a cryoprotectant (10 % v/v DMSO/ASW), and cryopreserved at −80 °C, a sample of seawater collected at the same time was also stored in the same way. For analysis, the samples were processed as described in section 6.3.2.1 with slight variation. Following rinsing in fresh sterile seawater, the rinsed sponge was dissected into small pieces (approximately 1mm × 1mm) using a sterile scalpel, and homogenized further using in a Stomacher® 400 circulator (Seward, UK). The resultant homogenate was suspended in 20 mL of sterile artificial seawater.

6.3.4.2 Culturing of associated bacteria

The homogenised sponge and collected local seawater were serially diluted (1:10) two times giving a total of 63 suspensions from 31 collected samples. 100 µL of each suspension was pipetted onto three different types of seawater-based agar AIA, SYP and MA (2216) making 189 plates, which were also repeated in duplicate. The solution was spread using a sterile spreader to give an even film over the plate before being wrapped in parafilm, to prevent the agar drying out, and incubated at 20 °C. Each plate was checked on a daily basis over the next three months and any colonies showing distinct morphological characteristics were removed with a sterile loop and streaked on to nutrient rich MA (2216). These plates were incubated at 25 °C for 2-3 days and single colonies were removed from the end of the

streaks using sterile loops and used to inoculate a solution of 1 mL sterile 30 % v/v (glycerol/ASW). These samples were cryopreserved at −80 °C and kept as a record ready for identification or grown on slopes of MA (2216) for immediate use, which were stored in a refrigerator at 2 to 8 °C. If the streaking process did not produce clear single colonies, the process was repeated until single colonies was achieved.

6.3.5 Large scale culturing of active colonies followed by three step solvent extraction

A seed culture of the chosen bacterium was defrosted and prepared by inoculating 1 mL culture in 50 mL of MB (2216) and incubating for 48 h in a shaking incubator at 30 °C and 200 rpm. 10 mL of seed culture was transferred into four 2 L Erlenmeyer flasks filled with 1 L of sterile MB (2216) to produce extra surface movement. The suspension was incubated for three days at 30 °C and shaken at 200 rpm. The resultant cultures were centrifuged (20 min, 4 °C, 3500 ×g) and the supernatant removed. The resultant pellet was extracted using the three solvent extraction method used for Welsh sponge material in section 4.3.1. This resulted in three extracts for each active colony, which was then tested for activity using the broth dilution method (section 4.3.4) and direct bioautographic TLC (section 4.3.2). Identification and dereplication was completed using direct TLC-MS and the inclusion into the molecular network formed following HPLC-MS/MS (section 5.3.6).

6.3.6 DNA extraction of pure and mixed cultures

An aliquot of 1 mL of sample (pure or mixed culture) was centrifuged (10 min, 4 °C, 2600 ×g) in a 1.5 mL micro-centrifuge tube and the supernatant removed. Another 1 mL of samples was added to the pellet and the process was repeated to effectively double the quantity of material. The resultant pellet was re-suspended with guanidine thiocyanate (GuT) (Sigma-Aldrich, UK) in bead beating tubes (Lysing Matrix E 2mL, MP Biomedicals, UK) and then lysed to release DNA, using a vortex (Genie® vortex) with adapter (MoBio Laboratories Inc., UK) for 10 min. The resultant sample was then incubated for 16 h at 55 °C. The following day the tubes with lysed material were centrifuged (10 min, 4 °C, 2600 ×g) and 400 µL of the supernatant was removed and transferred into the DNA extraction kit (Maxwell® 16 Tissue DNA Purification Kit, Promega, USA). The genetic material was extracted using 300 µL of elution buffer and a DNA extraction machine (Maxwell 16 Magnetic Particle Processor, Promega, USA) and then transferred to a fresh micro-centrifuge tube before being labelled and cryopreserved at −80 °C.

6.3.7 16S rRNA gene analysis of mixed samples

A selection of sponge samples and one sample of plant material (as a control) were sent to Research and Testing Laboratory (Lubbock, Texas, USA) for illumina MiSeq® 16S rRNA sequencing and analysis of their complete microbiome. The MiSeq data, was HV region V1-V2. The primers used were the 28F mixture and 519r, amplified using 30 cycles at 55 °C. The paired ends were merged using PANDAseq (Masella *et al*. 2012) with the PEAR algorithm and chimeras checked and removed using USEARCH61. Operational taxonomic units (OTU) were picked open reference, and matched using a 97% similarity (sequences were clustered using reference Greengenes 13_8 database. Those that did not match were clustered *de novo*). Alpha and beta diversity were completed using default parameters.

6.3.8 Polymerase chain reaction (PCR) assay prior to 16S rRNA gene analysis of pure cultures

PCR was performed by Biosciences Cardiff University, prior to 16S rRNA gene analysis of pure cultures to amplify specific regions ready for analysis. An outline of the conditions used is listed below:

A 50 µL reaction was completed for each sample using Promega UK reagents: GoTaq® G2 Flexi DNA Polymerase Kit, dNTP Mix (U1511) and PCR water (nuclease-free) (P1193) and 27F /907R primers (Table 6-1, Table 6-2). Thermo-cycling was completed with a Bio-Rad C1000 Thermocycler (Berkeley, USA).

Table 6-1 Formulation of PCR reaction mixture, using GoTaq® G2 Flexi DNA Polymerase Kit (Final volume 50 µl).

Component	Final Volume
GoTaq® G2 colourless Flexi Buffer	10 µl
$MgCL_2$ Solution (35 mM)	4 µl
Upstream primer 27F (10 pmol)	1µl
Downstream primer 907R (10 pmol)	1 µl
PCR water	32.75 µl
GoTaq® G2 Flexi DNA Polymerase (5u μl^{-1})	0.25 µl
Template DNA	1 µl
Total volume	50 µl

Table 6-2 Thermal-cycling profile used for PCR assay (Bio-Rad C1000 Thermocycler, Berkeley, USA).

Parameter	Denaturing		Annealing	Extension	Revert back to step 2 34×	Extension	Soak
	Step 1	Step 2	Step 3	Step 4		Step 5	Step 6
Temp °C	95	94	52	72		72	10
Time (mm:ss)	02:00	00:30	00:30	01:30		03:00	Hold

6.3.9 16S rRNA analysis of pure cultures

16S sequencing was completed for the PCR products (section 6.3.8) of pure cultures by Macrogen, Holland using an Applied Biosystems 3730XL sequencer (Life Technologies, USA). Files were received in fasta format before being sequenced on the forward strand (HV region V1-V5) and analysed with Basic Local Alignment Search Tool (BLAST) (NCBI, USA) with 97% similarity. Alignment was completed using the default settings in MEGA6 (http://www.megasoftware.net). The same sequences were analysed with BLASTN. The primers were 27F and 907R (both from Lane, 1991).

6.4 Results and discussion

6.4.1 Preliminary results and discussion

6.4.1.1 Cultivating bacteria from a cross-sectional sample of Cliona celata

Multiple samples of *Cliona celata* were collected via Scuba from the coast of Pembrokeshire, Wales in 2011. Samples were transferred in the water they were collected to the laboratory before being cryopreserved using DMSO (10 %) as a cryoprotectant. The first attempt of isolation of bacteria involved slicing a sponge samples in half, with a sterile scalpel, and then wiping the newly exposed sample on to the surface of a TSA plate and incubating at 37.5 °C. After 16 h a full lawn had grown and a sample was taking for identification using Gram staining. An unknown Gram-negative rod was visualised and then tested for antibacterial activity, using the deferred antagonism assay (section 6.3.3). The bacterial colony showed no inhibition of MSSA.

Although this was performed as a simple test to determine whether any bacteria from the sponge could be cultured for testing, it highlighted more robust techniques were needed for future tests. The agar chosen was not marine specific, therefore it did not provide the supporting environment needed for marine bacteria to grow such as the correct salinity and any potential nutrients and electrolytes that are normally present in seawater. This was resolved by using seawater based agars (Kennedy *et al*. 2009). The temperature used, although common for standard medically derived microbes, was not ideal for marine bacteria proliferation. This is because bacteria found in marine sponges in the UK thrive in temperatures between 10-16°C. For this reason, future incubation temperatures were limited to a maximum of 25°C.

6.4.1.2 In situ swabbing of C. celata

C. celata was swabbed *in sit*u at Martins Haven, Wales, which is part of the Wildlife Trust of South and West Wales (Skomer). It was decided, with staff from Skomer, that no sponges could be removed from this semi-protected area so a non-invasive technique of sampling was trialled (Figure 6-3). This technique involved removing a sterile swab underwater, and swabbing the outside of the sponge in an attempt to collect surface bacteria. A sample of seawater was taken from next to the sponge to use as a positive control to compare to the sponge swab.

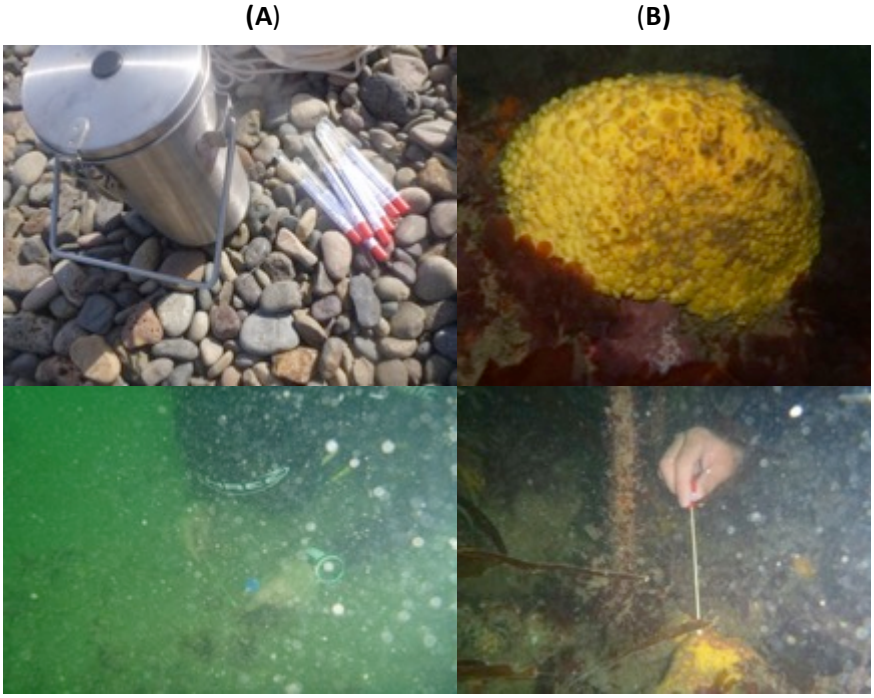


Figure 6-3 Digital images of: (A) Materials needed for swabbing, including sterile swabs and dry and insulated container filled with ice for storage of specimens; (B) *C. celata in situ*; (C) + (D) Swabbing *in situ*.

The samples were taken back to the laboratory in a chilled container, swabbed onto the surface of a marine agar plate and then incubated at 25 °C for up to one month.

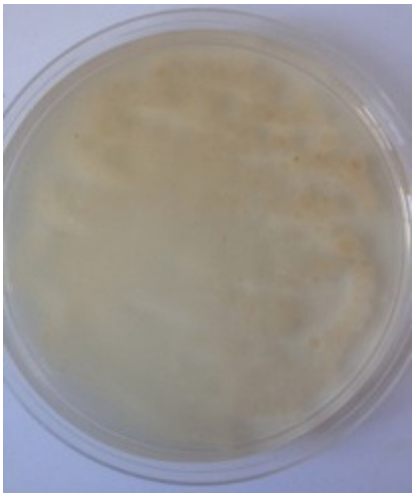


Figure 6-4 Digital image of overgrown agar plate from the swab of *C. celata* showing no colony differentiation.

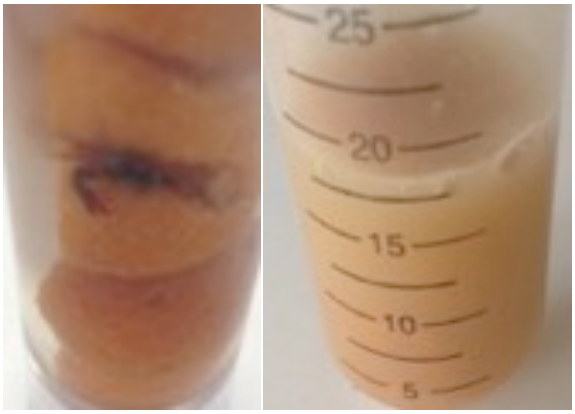
Both the seawater sample and swabs from *C. celata* produced overgrown plates, which could not be recovered (Figure 6-4). Gram staining of the sample showed a Gram-negative rod, similar to that seen in section 6.4.1.1. Since one organism produced such heavy growth, slow growing bacteria did not have the opportunity to compete and proliferate. For this reason, it was decided that all future samples would be serially diluted onto various different agars of

alternative nutrient compositions to give as many variations of conditions as possible to encourage growth of as many different bacteria as possible.

6.4.1.3 Test growth of bacteria from C. celata

6.4.1.3.1 Preparation of sample for bacterial cultivation

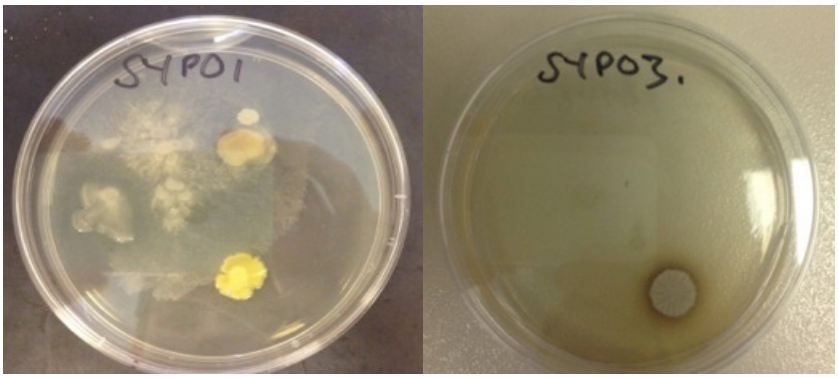
A sample of *Cliona celata* (section 6.3.2.1) was prepared for bacterial cultivation (Figure 6-5). Three samples were tested; the original fluid the sponge was stored in (O), the turbid solution following the first rinse (T), and the homogenised sponge (S). Each solution was diluted three times and labelled as such. A 100 µL aliquot of each solution was then pipetted on to three different seawater based agars and labelled as such (SW, MA and SYP).



(A) **(B)**

Figure 6-5 Digital images of: (A) Defrosted sponge sample of *C. celata*. A small crustacean was found inside the sponge, which was removed with tweezers; (B) Homogenised sample of *C. celata.*

The samples were then incubated at 20 °C and monitored for growth continuously over a period of months. When new colonies were found, they were removed with sterile loops and streaked onto a high nutrition agar plate and the plate they were found on to encourage growth. The agar plates were then visually inspected for the formation of distinct single colonies and other microbial growth on the agar (Figure 6-6).



(A) (**B)**

Figure 6-6 Digital images of bacteria cultivated from the original fluid *C. celata* sample was stored in (O), on SYP agar. (A) Mixed growth SYPO1; (B) unknown possibly fungal growth, SYPO3.

Upon isolation of individual colonies from the agar, this study identified two interesting points:

1. Bacteria proliferated in nutrient rich environments. This was particularly noticeable when the same colonies were streaked on both nutrient rich agar and low nutrient agar. It was important to streak on both agars as some bacteria might prefer starved conditions or if grown in low nutrient conditions, some bacteria might need nutrients previously provided by the sponge homogenate.
2. Growth of bacteria was heavier from sponge samples than that of the surrounding seawater. Other reports have previously identified similar occurrences with a difference of two orders of magnitude observed for some species (Friedrich *et al*. 1999), thus indicating that the sponge host provides an advantageous environment for bacteria to grow in comparison to the surrounding seawater the sponge was found in. This certainly supports the theory that the relationship between the sponge and the bacteria may be symbiotic.

Macerated *C. celata* was also added into anaerobic media and incubated alongside the agar plates to determine if any of the bacteria proliferated in anaerobic conditions and no growth was found. Therefore, this study focused solely on cultivating bacteria on aerobic agar plates.

6.4.1.3.2 Assessing the antibacterial activity of cultivated microbes using the deferred antagonism assay

The samples gathered were tested for antibacterial activity using the deferred antagonism assay (section 6.3.3) against MSSA. No activity was visualised although two samples were

kept for future testing. As many of the plates were still contaminated with fast growing bacteria or fungal strains, it was decided the experiment should be repeated in triplicate with added antibacterial and antifungal agents before attempting to pursue the activity of these samples any further.

6.4.1.4 Repeat of C. celata bacterial cultivation

Bacterial cultivation was attempted again for *C. Celata* allowing opportunity for more repeats and testing of alternative media including actinomycete specific agar. *Actinomycetes* and other *Streptomyces* species were targeted because they are a known source of unusual and active metabolites (Fenical and P. R. Jensen 2006). Amphotericin B and nalidixic acid were also added in an attempt to inhibit proliferation of fungal strains and fast growing bacteria, respectively. Amphotericin B was added to all agar plates and nalidixic acid was added to half the plates.

6.4.1.4.1 Media selected for repeat experiment on C. celata

The media was chosen to provide as many different conditions as possible to encourage the growth of unusual bacteria: MA (Marine agar), MAN (Marine agar + Nalidixic acid), SYP (SYP agar), AIA (Actinomycete isolation agar), AIAN (Actinomycete isolation agar + Nalidixic acid).

6.4.1.4.2 Isolation of pure cultures from C. celata sample

Altogether, 21 cultures were isolated to single colonies. Single colonies were then removed from each plate with a sterile loop and used to inoculate 10 mL of SYP broth. These suspensions were then incubated for two to three days until growth was visualised. Upon visualisation of growth, sterile glycerol (30% v/v) was added to each of the broth mix as a cryoprotectant before the samples were cryopreserved at −80 °C prior to activity testing.

6.4.1.4.3 Assessing the antibacterial activity of microbes, cultivated from the host sponge C. celata, using the deferred antagonism assay

Samples were partially defrosted and were tested for activity using the deferred antagonism assay (section 6.3.3) against MSSA. No activity was clearly identified and it was decided the assay should be repeated as the agar spiked with MSSA did not provide an even growth and thus the results were inconclusive. None of the isolated strains were active upon repeat of the experiment and therefore, no bacteria were identified for 16S rRNA analysis.

6.4.2 Isolation of individual colonies of bacteria from Welsh marine sponge samples

A more targeted study was then completed on the samples collected in varying environments around Wales. All agar plates contained an antibacterial and antifungal agent to prevent unwanted, fast growing, microbe proliferation and AIA was used to target the active compound rich actinomycetes. Microbial colonies were successfully grown on various marine specific agar plates and showed a large variation in appearance ((A), Figure 6-7). Colonies were selected for streaking based on interesting or unusual morphologies or if they showed inhibition or clearance zones. The chosen colonies were then streaked on to a plate, and re-streaked until visually pure colonies were formed ((B, C and D**)**, Figure 6-7).

(A) (**B)**

(C) (**D)**

Figure 6-7 Digital images of bacteria cultivated from marine sponge samples, in various stages of cultivation and isolation. (A) General mix of cultures showing a large variation in appearance. (B) White raised appearance of single colonies formed immediately on first streaking cycle (C) Bright orange bacterium colonies showing typical streaking pattern. (D) Mix of bacterium separated via streaking. Any mixed plates were then re-streaked to ensure purity.

6.4.3 Analysis of the complete biome of bacterial species found on Welsh marine sponges using 16S rRNA gene analysis

Analysis of the complete microbiome of chosen species was performed to allow comparison to the identification of pure cultures and to potentially provide extra ecological information for future collection of sponges. Images of each sponge *in situ* are presented in section 4.4.2.

Sample 4.1 was a pure culture isolated from sample 4 and was tested as a control (Table 6-5).

The taxa summary presented in Figure 6-8 shows a summary of the bacterial families present on each sponge in a percentage based stacked column graph, with each different colour band representing a different bacterial family. By narrowing the class bacteria down to family, clear differences were observed between bacterial family and parent sponge species and environment. The first observation made was that the most commonly detected family of microbe was *Proteobacteria*. This was expected as they are known nitrogen fixing bacteria, which may benefit sponge metabolism (Chen *et al*. 2003; Freeman and Thacker 2011) and they are commonly recovered from marine sponges (Webster *et al*. 2001). The common occurrence of *Proteobacteria* found in this gene analysis study may also explain the isolation of an overwhelming number of gram-negative rods in the preliminary results (section 6.4.1).

Table 6-3 Identification key for complete biome of bacteria found on marine sponge. ((c)= competitive environment, (s) = collected via SCUBA). The ID numbers refer back to the collection number displayed in section 4.4.2.

ID number	Species	ID number	Species
4	*Hymeniacidon perleve*	**16**	*Halichondria panicea*
6	*Hymeniacidon perleve*	**19**	*Halichondria panicea* (c)
7	*Hymeniacidon perleve*	**20**	*Dysidea fragilis*
8	*Hymeniacidon perleve*	**28**	*Halichondria panicea* (s)
9	*Amphilectus fucurom*	**29**	*Suberites ficus (s)*
10	*Plant material*	**30**	*Amphilectus fucorum* (s)
13	*Halichondria panicea*	**4.1**	*Hymeniacidon perleve*
14	*Halichondria panicea* (c)		

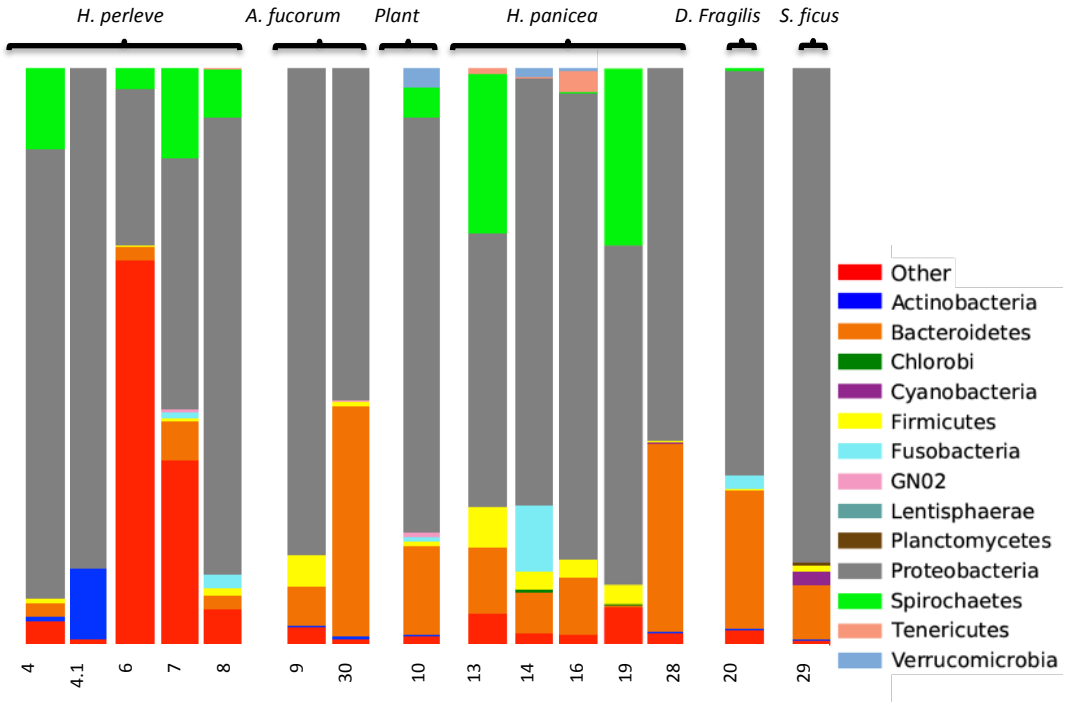


Figure 6-8 Complete taxa summary of bacterial families found on Welsh marine sponges identified through 16S rRNA analysis. (Sample and parents sponge key = Table 6-3).

A noteworthy difference observed in results depicted in Figure 6-8 is the appearance of *Spirochaetes* within samples, as they only appear in detectable numbers in *H. perleve* (samples 4, 6, 7 and 8) or *H. panicea* (samples 13 and 19) found in direct competition with *H. perleve* (Table 6-3) and only in samples found in shallow waters. While scarcely recorded in *H. panicea* in an isolated environment (samples 14, 16 and 28), when in competition with *H. perleve* (13 and 19), *Spirochaetes* were actually found in larger proportions than in isolated *H. perleve* (samples 4, 6, 7 and 8). This may have arisen from cross contamination upon collection, as some competitive samples were particularly well bound to the substrate, or the combination of the two sponges provided a superior environment for the *Spirochaetes* to proliferate.

A further interesting observation was the difference in proportion of *Bacteroidetes* identified in different sponge samples. Their proportions were largest in the samples collected via scuba, which indicated that they were most likely salt dependent, as they were found deeper in the ocean. It is known when searching for antibacterial metabolites that some bacterial species will only produce active metabolites in the presence of sea salt, sodium chloride (Okami *et al*. 1976). Another interesting observation is that the family *Actinobacteria*, which contain the targeted genus' of *Streptomyces* and *Actinomycetes* were poorly represented in all the crude taxa samples, thus accounting for the specialist methods of cultivation used to

isolate them *e.g.* Actinomycete isolation agar and nalidixic acid to prevent fast growing bacteria.

While the distinct sponge-microbe-species specificity interactions identified in this project have never been previously documented, general species specificity interactions are not a novel phenomenon and have been documented before, although it is not representative of all sponge-microbe relationships (Wilkinson *et al*. 1981). An example of this previously identified specificity is that of a dominant *Rhodobacter* species, which during a study of multiple sponge species was only isolated from *Halichondria panicea* (Althoff *et al*. 1998), one of the sponge species studied as part of this research project.

Figure 6-9 shows a more complex variation of the taxa summary pictured in Figure 6-8 displaying the taxonomic species of the bacteria identified rather than the family. Figure 6-9 shows all the different bacterium species present on each sponge in a percentage based stacked column graph. The key for this complicated figure is found in the appendix II but in summary, each colour-coded band represents a specific bacterial species. Sample 4.1, which was presumed to be a pure culture, contains three different species of bacteria. When compared to the complete microbiome from the sponge (sample 4, Table 6-3), there was only one obvious match in bacterium and this was found in minor proportion in the sponge microbiome gene analysis. This is not particularly surprising as the cultures were grown in a mixed culture so purification may be difficult especially if the bacterium commonly co-exist. It was also to be expected to find something not commonly found on the sponge as fast growing species and maybe the majority of the strains identified were purposely inhibited by the introduction of nalidixic acid in the agar plate. It is also possible that some of the more commonly detected bacterium in the crude samples in Figure 6-9 may not be culturable by the methods employed. As previously mentioned, it is recognised that less than 1% of bacteria found on sponge are commonly culturable (Amann *et al*. 1995).

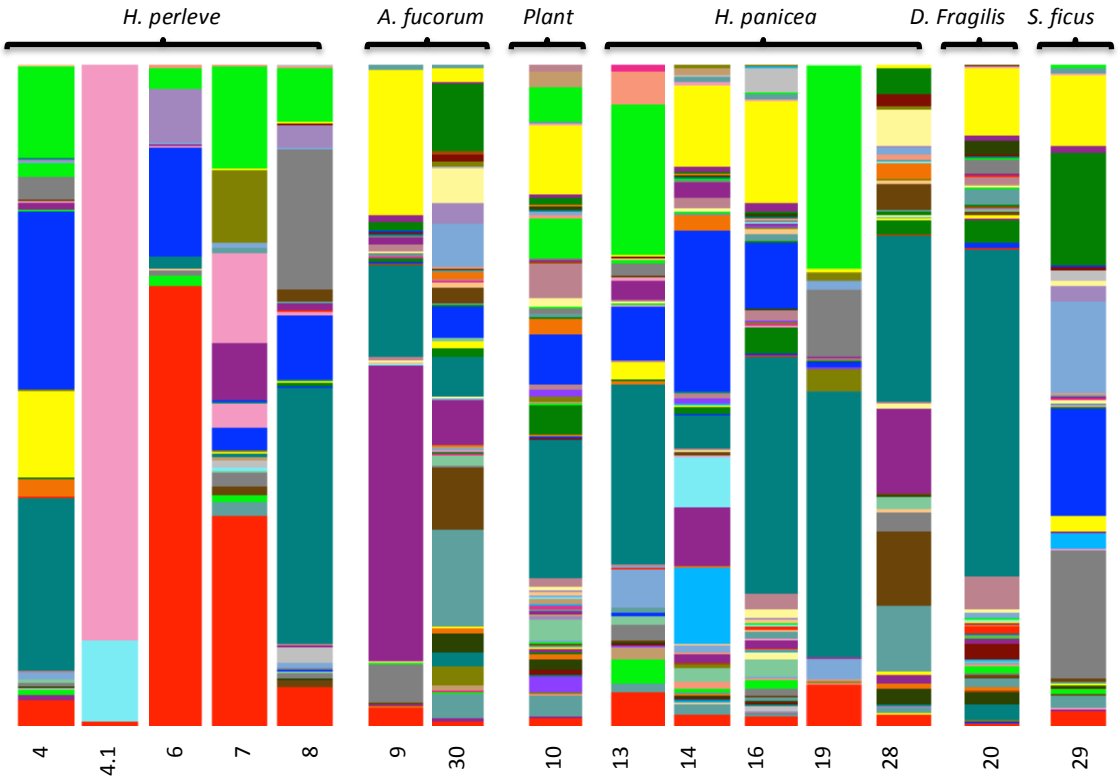


Figure 6-9 Complete taxa summary of bacterial species found on Welsh marine sponges identified through 16S rRNA analysis. For the key to this complex figure, see Appendix. Sample and parent sponge species key = Table 6-3.

6.4.4 Assessing the antibacterial activity of individual colonies of bacteria isolated from Welsh marine sponges using the deferred antagonism assay

All samples isolated to single colonies were tested for antibacterial activity using the deferred antagonism assay (section 6.3.3). Some examples are shown in Figure 6-10.

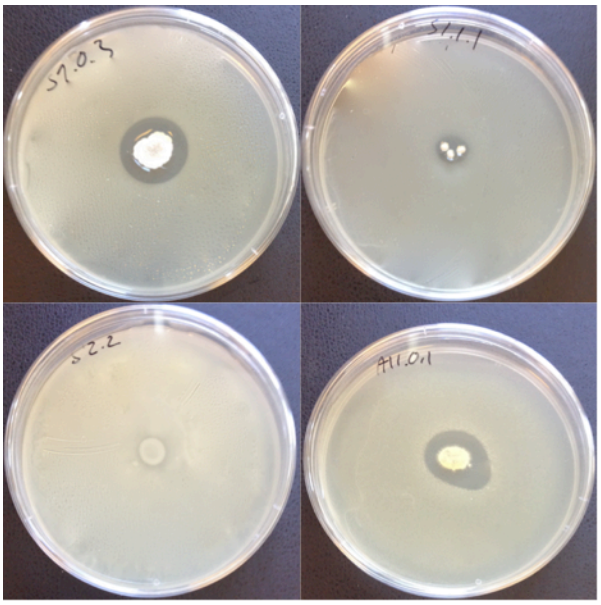
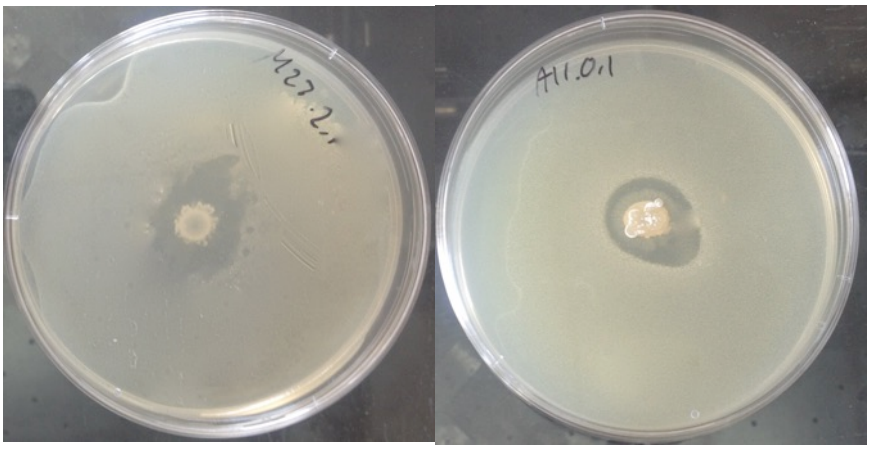


Figure 6-10 Digital images of deferred antagonism assay against MRSA. Zones of inhibition around pure cultures of sponge-derived bacteria indicate activity against MRSA. (A) - (D) A variety of isolated cultures showing differing appearance and differing size and shape of zones of inhibition.

Due to some of the unusual sizes and shapes of the colonies formed (Figure 6-10), antibacterial activity determination could not be completed in the cross sectional diameter manner as previously utilised for the well method and disc diffusion method (section 4.3.3). Inhibition was instead classified as follows: if a zone was formed at an average of more than 4mm from the colony, it was recorded as +++; in between 2mm and 4 mm, it was recorded as ++ and less than 2mm, it was recorded as + (Table 6-4). A similar method of classification of results of the deferred antagonism assay was employed by Baker (2009), but included no scale of activity with either active or not active (+/-) recorded. The method employed by this study used similar classification to that of the disc diffusion assays reported by Amade (Amade *et al*. 1987; Amade *et al*. 1982). Each isolate was named in the following manner: The first letter represented the agar used for cultivation A = AIA, S = SYP and M = MA (2216). The adjacent number represents the sample ID from collection, all pictures can be found in section 4.4.2. The next number represents the dilution factor (1:10) 0 - 2. And any further numbers occurred through isolation of more than one colony from an agar plate.

Table 6-4 Antibacterial activity of isolated single colonies against MRSA. Non-sponge material was plant material taken from the site of collection as a control. (+++ Denotes zone of inhibition > 4mm, ++ denotes area of inhibition ≥ 2mm and + denotes area of inhibition < 2mm from the colony of cultivated microbe).

Sample ID	Sponge species	Intensity of activity	Sample ID	Sponge species	Intensity of activity
A11.0.1	*H. panicea*	+++	**S3.1.5**	*H. perleve*	+++
S2.2	*H. perleve*	+	**S3.1.4**	*H. perleve*	++
A27.2.2	*H. panicea*	+++	**S3.1.3**	*H. perleve*	++
S3.1.1	*H. perleve*	++	**A27.0.1**	*H. panicea*	++
S1.1.1	*H. perleve*	++	**A18.1.2**	*H. panicea*	+
A26.0	*H panicea*	+++	**A31.1.2**	*Sea water*	++
S26.1.4	*H. panicea*	++	**M27.0.2**	*H. panicea*	++
S22.1	*H. panicea*	+	**A18.1.2**	*H. panicea*	++
S7.0.3	*H. perleve*	+++	**A31.1.2**	*Sea water*	+
A9.0.2	*A. fucorum*	++	**M7.2.1**	*H. perleve*	++
A17.1.1	*Non sponge*	++	**A1.1.1**	*H. perleve*	+
A11.2.1	*H. panicea*	+++	**A22.1**	*H. panicea*	++
M27.2.1	*H. panicea*	+++	**S26.1.1**	*H. panicea*	+
A9.2	*A. fucorum*	++	**A27.2**	*H. panicea*	+
S7.0.2	*H. perleve*	++	**A5.0**	*H. perleve*	+
S10.2.2	*Non-sponge*	+	**S7.2.3**	*H. perleve*	+
S10.2.1	*Non-sponge*	+	**A25.0**	*H. panicea*	+



(A) **(B)**

Figure 6-11 Digital image of deferred antagonism assay with isolated colonies (A) M27.2.1 (*H. panicea*) and (B) A11.0.1 (*H. panicea*) with zones of inhibitions against *E. coli.*

A large variety of activity was found with 18% of all single colonies isolated displayed activity against MRSA. The colonies A11.0.1 (*H. panicea*), M27.2.1 (*H. panicea*), A26.0 (*H. panicea*) and S7.0.3 (*H. perleve*) not only showed high (+++) activity against MRSA but were also the only strains that displayed antibacterial activity against *E. coli* (Figure 6-11). Although it is promising that a large number of colonies displayed antimicrobial activity, this study did not examine a complete random selection of the bacteria present on a host sponge. Colonies

were selected deliberately based on a variety of appearance and an antibacterial agent was added into the agar to prevent fast growing bacteria. While this was useful in selective growth, it was not particularly useful for identification of the average make up of symbiotes found on a sponge. Therefore, direct comparison of the number of active bacterial strains present between individual sponge species cannot be made. The parent species was only important for this study when attempting to identify the compounds responsible for observed activity and dereplication.

6.4.5 Identification of microbes responsible for antibacterial activity using 16S rRNA sequencing

Selected samples with confirmed antibacterial activity against MRSA were then identified using 16S rRNA sequencing. The untrimmed sequences from the 16S analysis of pure cultures were analysed by the Basic Local Alignment Search Tool (BLAST) with alignment also run to generate a neighbour-joining tree (Table 6-5, Figure 6-12).

Table 6-5 Identification key for taxa summary tree. Previously uncultured samples highlighted in bold. Non-sponge material was plant material taken from the site of collection as a control.

Number	Sponge species	Sample ID	Number	Sponge species	Sample ID
CH1	*H. perleve*	S1.1.1	CH10	*H. panicea*	A11.0.1
CH2	*H. perleve*	**S2.2**	CH11	*H. panicea*	**A11.2.1**
CH3	*H. perleve*	S3.1.1	CH12	*Non-sponge*	A17.1.1
CH4	*H. perleve*	**S3.1.3**	CH13	*H. panicea*	A18.1.2
CH5	*H. perleve*	S3.1.4	CH14	*H. panicea*	S22.1
CH6	*H. perleve*	S7.0.3	CH15	*H. panicea*	A26.0
CH7	*H. perleve*	S7.0.2	CH16	*H. panicea*	S26.1.4
CH8	*A. fucorum*	A9.0.2	CH17	*H. panicea*	A27.2.2
CH9	*A. fucorum*	A9.2	CH18	*H. panicea*	M27.2.1

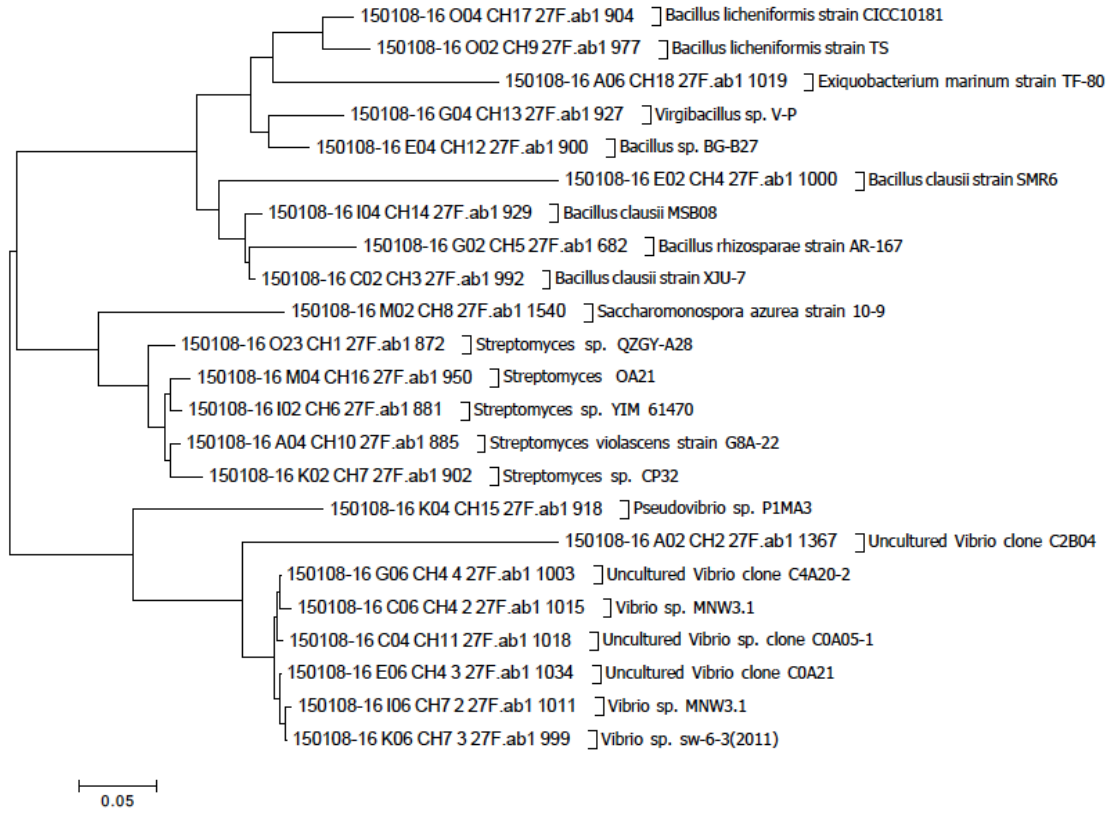


Figure 6-12 Taxa summary tree for pure active isolates identified using 16S rRNA sequencing. Sample ID key shown in Table 6-5.

Analysis of the taxa summary tree revealed that 18 'single colonies' were identified using 16S gene sequencing (Figure 6-12). The results show that three major phylogenetically different clusters of bacteria were isolated; *Vibrio sp*., *Streptomyces sp*. and *Bacillus sp.*, appearing in 8, 6 and 9 samples, respectively. None of the species identified matched those of the complete microbiome (Figure 6-9) suggesting the methods employed were successful in

cultivating more unusual bacteria found on the sponge. Sample CH4 and sample CH7 both showed a presence of a mix of bacteria, which probably occurred through contamination or incomplete separation during the streaking process. Bacterial samples may not have separated during the streaking process due to a close relationship formed *in situ*, a similar observation was noted during the examination of the complete biome found on samples (section 6.4.3). The true source of activity from these bacterial samples could be uncovered by further re-streaking and repeating 16S rRNA gene sequencing and activity testing but it was decided that this would not be pursued due to the further objectives of this project. It is also possible that the activity was actually caused by a synergistic relationship between the bacteria present.

Four previously uncultured organisms where successfully cultured and identified using 16S sequencing. They also displayed previously unreported antibacterial activity. These were all uncultured vibrio clones C2B04, C4A20-2, C0A21 and C0A05-1, respectively. The isolation of some of the bacterial strains was particularly interesting such as *Bacillus licheniformis*, which is normally associated with terrestrial organisms and is commonly found in bird feathers, although the strain CICC10181 has previously been associated with marine bacteria (Devi *et al*. 2010). Multiple members of the target family of *Streptomyces* were also isolated including one species of actinomycete *Saccharomonospora azurea* strain 10-9, which was cultivated on actinomycete isolation agar. *Saccharomonospora azurea* is normally associated with soil samples (R. Hu 1987) and although it may be possible that it actually originated from the silt heavy water from which the samples were collected. In this case, it was directly isolated from the sponge sample.

The 16S rRNA results presented were from untrimmed sequences. The results could be improved by the use of a program like *Geneious* or a sequence editor, in which the sequences can be trimmed in places like the forward primer and noisy areas at the end to result in a cleaner sequence. On this occasion, it was decided that identification was clear enough for the scope of this project.

6.4.6 Large scaled production of antibacterial bacteria strains

In order to attempt chemical profiling, two samples were chosen for a scaled-up production, based on their antibacterial activity (section 6.4.4) and were re-named B11 and B27 for ease of labelling. Both strains were isolated from *Halichondria panicea* and collected during a low spring tide.

A11.0.1 or B11 *Streptomyces violascens* strain G8A-22, is of the same family as Actinomycetes which were specifically targeted for their metabolite production. A similar activity based chemical profiling was completed on a bacterium of the same genus in 2014 (Viegelmann *et al*. 2014).

M27.2.1 or B27 *Exiguobacterium marinum* strain Tf-80, is a marine specific Bacillus discovered in 2005 (I.-G. Kim *et al*. 2005), which has not been previously chemically profiled.

Samples were then mass cultured and extracted (section 6.3.5), in the same manner as the sponge samples, using three solvents of increasing polarity hexane, acetone then methanol (section 2.2.3). The resultant fractions were then labelled with the first initial of the solvent used for extraction, for example BH11, BA11 and BM11 representing the hexane, acetone and methanol extracts of B11.

6.4.7 Assessing the antibacterial activity of bacterial extracts against clinically relevant bacteria

Upon separation using the sequential extraction method, extracts were tested for antibacterial activity using the microdilution method to calculate MIC and MBC (section 4.3.4, The digital images of the determination of the MIC/MBC of acetone bacterial extracts are also displayed in this section).

A MIC was only observed in the acetone extracts, both methanol extracts showed no inhibitory affect at the concentrations tested. BA11 and BA27 displayed MICs of 1.28 mg mL^{-1} and 2.56 mg mL^{-1}, respectively and were comparable to some of the whole sponge extracts. It must be noted however that some sponge samples displayed greater activity towards MRSA with one sample of the parent sponge *H. panicea* (2w15) demonstrating a MIC of 0.16 mg mL^{-1}.

MBC was also tested by pipetting samples from the wells onto MHA. It was impossible to calculate the MBC of BA27 as bacteria grew at all concentrations tested. However, BA11 (*Streptomyces violascens* strain G8A-22) displayed an MBC of 1.28 mg mL^{-1}, which suggests that the compounds responsible for the activity were bactericidal. BA11 also displayed activity against *E. coli,* which was measured to be 2.56 mg mL^{-1} (MIC) but displayed no bactericidal activity against *E. coli* at the concentrations tested (Table 6-6).

Table 6-6 Summary of the antibacterial activity of the acetone extracts of. BA27 (*Exiguobacterium marinum* strain Tf-80) and BA11 (*Streptomyces violascens* strain G8A-22). MIC and MBC against MRSA (NCTC 11939) and E. coli (12210) displayed. If no MIC or MBC was observed recorded as (-).

Bacterial Strain	MRSA (NCTC 11939)		*E. coli* (NCIMB 12210)	
	MIC (mg mL^{-1})	MBC (mg mL^{-1})	MIC (mg mL^{-1})	MBC (mg mL^{-1})
***Streptomyces violascens* strain G8A-22 (BA11)**	1.28	1.28	2.56	-
B27 *Exiguobacterium marinum* strain Tf-80 (BA27)	2.56	-	-	-

6.4.8 Direct bioautographic TLC-MS as a dereplication strategy for antibacterial bacterial extracts

As described previously (section 5.3.3), two duplicate TLCs were subjected to our screening workflow. The methanol extracts of both BA11 and BA27 contained compounds that displayed some potential activity on the baseline but this was not particularly clear. However the acetone extracts showed clear activity across the plate suggesting several compounds were responsible for activity (Figure 6-13). The use of the bioautographic TLC method was validated when the plate (visualised under UV) was compared to that of overlaid bacteria, where it was observed that compounds or fractions identified as Ba11.4 and Ba27.2, both demonstrated antibacterial activity but may have been missed if separated using HPLC as they were not visible at UV wavelength of 254 nm (Figure 6-13).

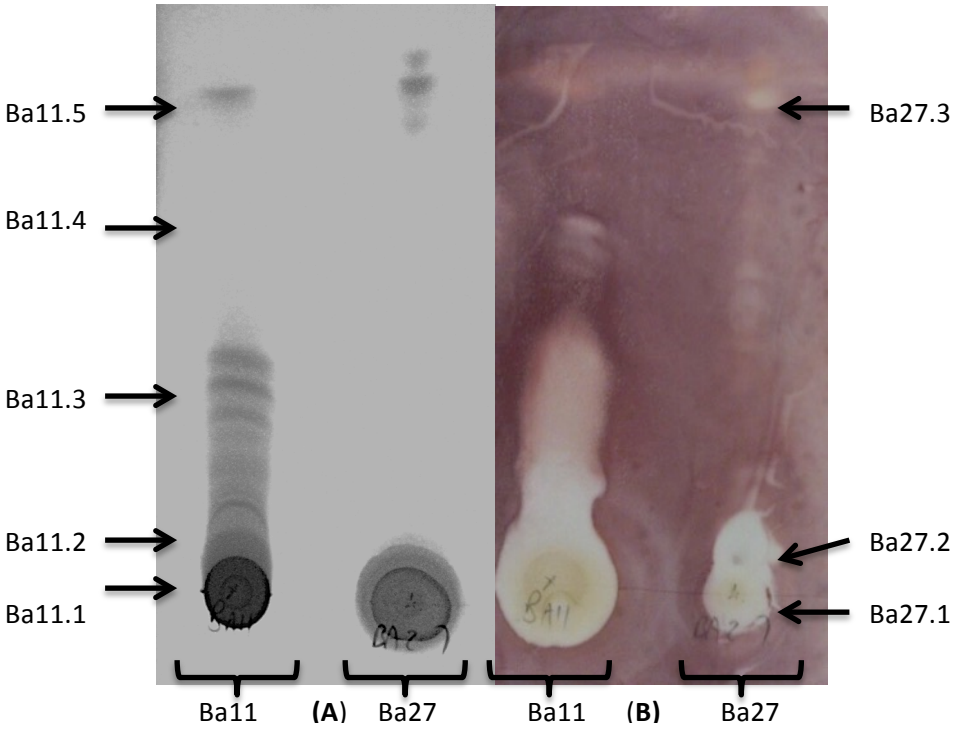


Figure 6-13 Visualisation of antibacterial activity against MRSA of acetone extracts of BA11 (*violascens* strain G8A-22) and BA27 (*Exiguobacterium marinum* strain Tf-80) using the bioautographic overlay TLC assay. (A) TLC plate under UV light 254nm; (B) Growth inhibition visualised using the metabolic stain INT.

TLC-MS analysis was completed on the duplicate plate to obtain an observed mass for each area of activity. Predicted masses of the expected ions were calculated and displayed in Table 6-7 to allow cross-reference with the marine specific database *MarinLit* as a method of dereplication.

Table 6-7 Summary of bioautographic TLC – MS for BA11 *Streptomyces violascens* strain G8A-22 and BA27 *Exiguobacterium marinum* strain Tf-80, both from parent sponge of *H. panicea* species with Rf 6:4 (hexane : acetone), estimation of activity, observed mass value of active compound, observed isotope patterns and the predicted mass values. Predicted mass was estimated by calculating difference in mass that the formation of the selected ion would cause when compared to the observed mass. Predicted masses are coloured based on their classification post dereplication. (Black = Known compound matched to sponge, Red = Novel compound, Blue = Possibly novel compound).

Sample	Fraction name.	Estimated activity	Rf of Active spot	Observed mass value	Predicted mass values			Isotope pattern
					$(M+H)^+$	$(M+Na)^+$	$(2M+Na)^+$	
BA11	*BA11.1*	*+++*	*0*	304.3	303.3	281.3	140.7	
	BA11.2	*++*	*0.11*	379.2	378.2	356.2	178.1	
				326.4	325.4	303.4	151.7	
	BA11.3	*++*	*0.41*	357.3	356.3	334.3	167.2	
				581.3	580.3	558.3	279.2	
	BA11.4	*+*	*0.67*	357.3	356.3	334.3	167.2	
				413.4	412.4	390.4	195.2	
	BA11.5	*+*	*0.97*	685.5	684.5	662.5	331.3	
				1347.1	1346.1	1324.1	662.1	*Dimer?*
BA27	*BA27.1*	*++*	*0*	553.5	552.5	530.5	265.3	
	BA27.2	*++*	*0.11*	779.0	778.0	756.0	378.0	
	BA27.3	*+*	*0.97*	663.0	662.0	640.0	320.0	

Analysis of the TLC-MS data was completed using *MarinLit* as the primary source of information and a direct comparison to *SciFinder* was also completed. All masses were checked against the parent sponge genus, starting at species to find the most likely compounds.

6.4.8.1 Bacterial extract BA11 Streptomyces violascens strain G8A-22

The acetone extract of a culture grown from the Welsh sponge *Halichondria panicea* (BA11) and identified as *Streptomyces violascens* strain G8A-22, showed the most growth inhibitory activity from all bacterial extracts tested in both the bioautographic overlay method and MIC testing (section 6.4.7). The most active spot (BA11.1), identified in the bioautographic assay, was that on the baseline, had an observed mass of *m/z* 304.3, suggesting a parent mass of 303.3 (M + H) confirmed by the presence of an $(M + Na)^+$ peak at *m/z* 326.4.

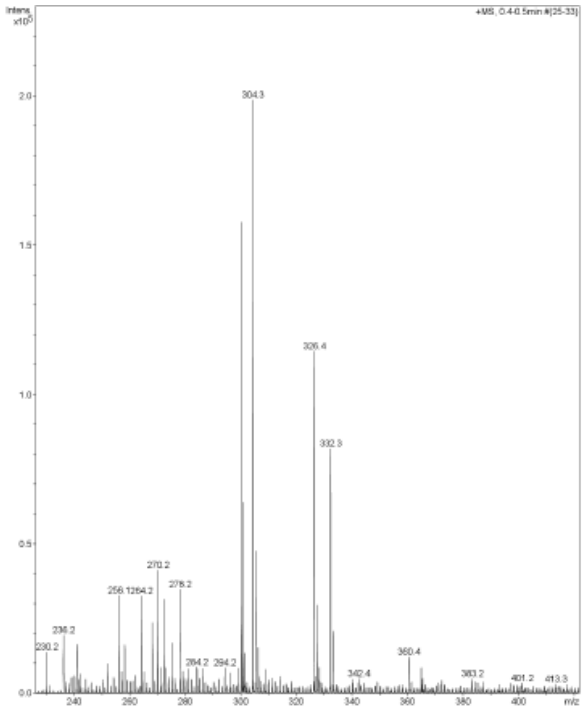


Figure 6-14 Mass spectrometry analysis of BA11.1 (*Streptomyces violascens* strain G8A-22) isolated from *H. panicea*, showing $(M + H)^+$ and $(M + Na)^+$ peaks for M=303 species.

A search of *MarinLit* identified the most likely structures as Netamine B a tricyclic guanidine alkaloid or the alkylpyrrole carboxaldehyde derivative depicted in Figure 6-15. Previously, both compounds have only been isolated directly from the same taxonomic class of sponge (Sorek *et al*. 2006; Venkatesham *et al*. 2000) and not from the associated bacteria. This suggests that either the associated bacteria cultivated from the sponge were independently producing these compounds or the mass could represent a novel compound.

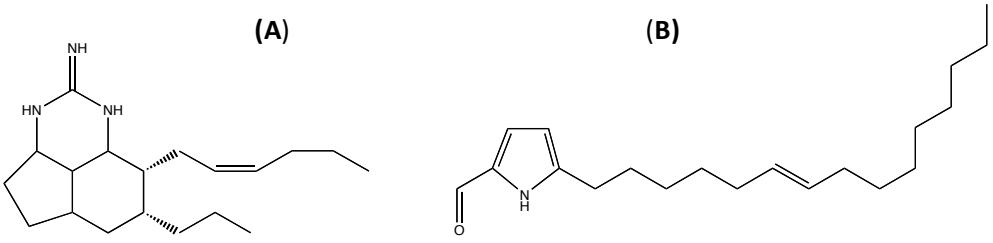


Figure 6-15 Chemical structures of (A) Netamine B (7R,8R)-8-((Z)-hex-2-en-1-yl)-7-propyldecahydrocyclopenta[de]quinazolin-2(3a1H)-imine; Formula: $C_{19}H_{33}N_3$; Mass: 303.27 and (B) (E)-5-(pentadec-6-en-1-yl)-1H-pyrrole-2-carbaldehyde, Formula: $C_{20}H_{33}NO$; Mass: 303.26.

Another compound identified in the literature that could possibly account for the mass of 303.3 $(M + H)^+$ isolated from marine organisms is cycloxenicane (Figure 6-16), which has

previously been isolated from brown algae (Ioannou *et al*. 2009). Algae are also rich in associated bacteria, suggesting a similar biosynthetic origin.

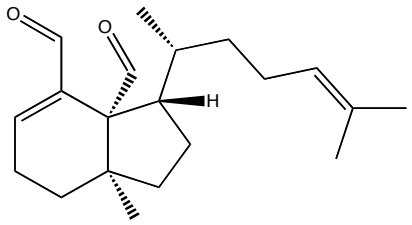


Figure 6-16 Chemical structure of cycloxenicane, (3S,3aR,7aR)-7a-methyl-3-((R)-6-methylhept-5-en-2-yl)-2,3,3a,6,7,7a-hexahydro-1H-indene-3a,4-dicarbaldehyde; Formula: $C_{20}H_{31}O_2$; Mass: 303.230.

The *Streptomyces violascens* extract BA11.2 produced two different masses to consider *m/z* 326.4 and 379.2. Only one literature match occurred for 378.2 $(M + H)^+$, a compound isolated from the same taxonomy family as *Halichondria panicea*. The steroid cholest-4,6,8(14),22E-tetraen-3-one was isolated directly from the sponge (Ciminiello *et al*. 1989) rather than the bacteria. It is possible that this compound originated from the bacteria associated with the sponge, and therefore may have consequently appeared in the sponge extraction however this is unlikely because although bacteria are known to produce complex terpenoids they very rarely produce steroids. Another method of approach was to search the bacterial species the compound originated from and upon searching *MarinLit*, actinofuranone A (Figure 6-17), which was originally isolated from a marine derived bacterium related to *Streptomyces* (Cho *et al*. 2006), and R-indomycinone, a pluramycin derivative originally isolated from an *Actinomycete* (Schumacher *et al*. 1995), appeared as potential candidates.

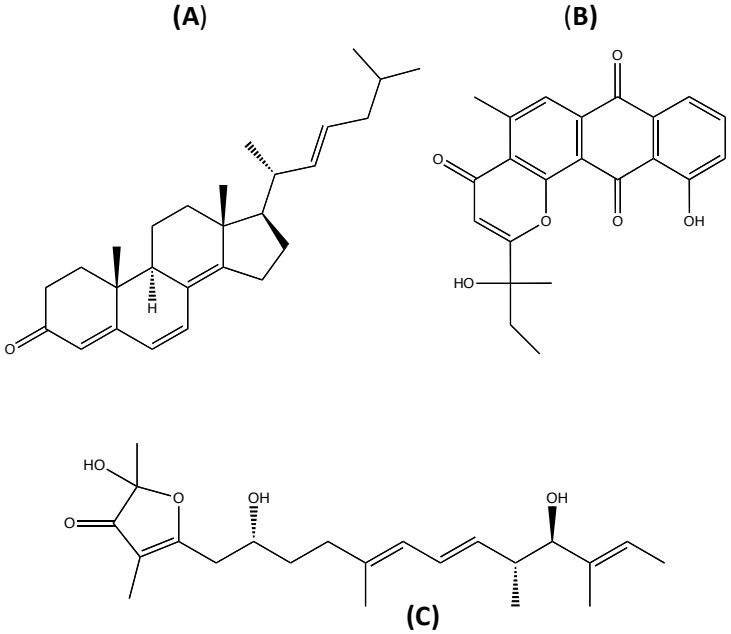


Figure 6-17 Chemical structures of (A) Cholest-4,6,8(14),22E-tetraen-3-one; Formula: $C_{27}H_{38}O$; Mass: 378.29, (B) 11-hydroxy-2-(2-hydroxybutan-2-yl)-5-methyl-4H-naphtho[2,3-h]chromene-4,7,12-trione; Formula: $C_{22}H_{18}O_6$; Mass: 378.11 and (C) Actinofuranone A, 5-((2R,5E,7E,9R,10R,11E)-2,10-dihydroxy-5,9,11-trimethyltrideca-5,7,11-trien-1-yl)-2-hydroxy-2,4-dimethylfuran-3(2H)-one; Formula: $C_{22}H_{34}O_5$; Mass: 378.24.

Although the $(M + H)^+$ species was considered the most likely ionisation form of a molecule using positive mode ES-MS it is also possible $(M + Na)^+$ may have occurred and this predicted ion mass was checked for each mass identified. For example, BA11.2 produced the mass of *m/z* 378.2 $(M + H)^+$, which proposed three natural products that may have been the compound isolated (Figure 6-17). However, if the ion produced was $(M + Na)^+$, a mass of *m/z* 356.21 is predicted, which matches the aromatic sesquiterpinoid Panicein-C (Figure 6-18), originally isolated from *Halichondria panicea* (Cimino *et al*. 1973). Although terpenoids are not normally associated with bacteria recent research has shown they are able to produce them, notably sesquiterpenes from Streptomyces and Actionmycetes (Cane and Ikeda 2012). Once more, it is possible that associated bacteria found on the sponge were responsible for the production of this compound rather than the sponge itself, in a similar manner to the brominated biphenyl ethers formerly attributed to the sponge *Dysidea. Sp*, which are now known to be produced by an associated bacterium (Elyakov *et al*. 1991).

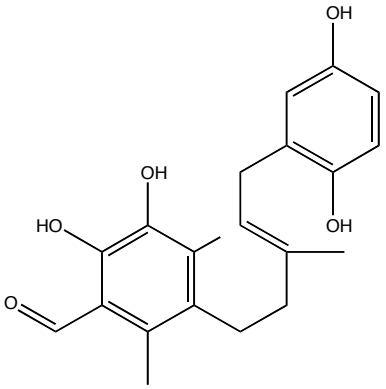


Figure 6-18 Chemical structure of Panicein-C; Formula: $C_{21}H_{24}O_5$; Mass: 356.16.

The mass of *m/z* 325.4 $(M + H)^+$ produced no matches when *MarinLit* and *SciFinder* were searched using the taxonomic information of both potential parent species. This indicated that it has never been extracted from marine sponge or associated bacteria class. One hit that appeared on *MarinLit* relates to semiplenamide C, a fatty acid amide isolated from a marine cyanobacterium collected in Papua New Guinea (Han *et al*. 2003). However this was a particularly tenuous link, therefore in this study the compound represented by the peak at 325.4 was considered to be novel.

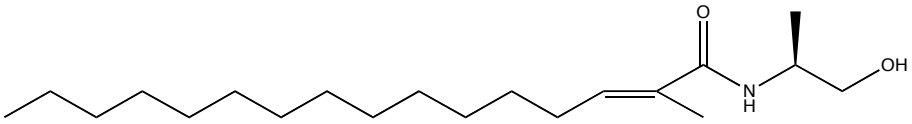


Figure 6-19 Chemical structure of Semiplenamide C, (S,E)-N-(1-hydroxypropan-2-yl)-2-methylhexadec-2-enamide; Formula: $C_{20}H_{39}NO_2$; Mass: 325.30.

Finally, antibacterial extract BA11.3 produced *m/z* 356.3 $(M + H)^+$; which suggested the structure of octalactin A, a cytotoxic eight-membered-ring lactone originally isolated from a marine bacterium *Streptomyces sp.* (Tapiolas *et al*. 1991). However this mass could also represent Panicein-C, m/z 356.2 $(M + H)^+$, which has been previously isolated from the host sponge species (Cimino *et al*. 1973) (Figure 6-18). The second mass found in Ba11.3 *m/z* 580.3 was originally thought to represent glycosylated macrolactin A1 or B1, which have been previously isolated from *Streptomyces*. Another possibility not suggested by the *MarinLit* search was an avermectin derivative which was found by examining a paper which reported the secondary metabolites of bacteria (Viegelmann *et al*. 2014). In this paper avermectin was isolated alongside some derivatives from bacteria, of the same family, originally associated to a marine sponge, making it one of the best comparisons possible. The structures from this paper were considered along with potential derivatives, and as one of

the precursors of avermectin displays a molecular weight of 582.3 and must be considered a possibility (Figure 6-20).

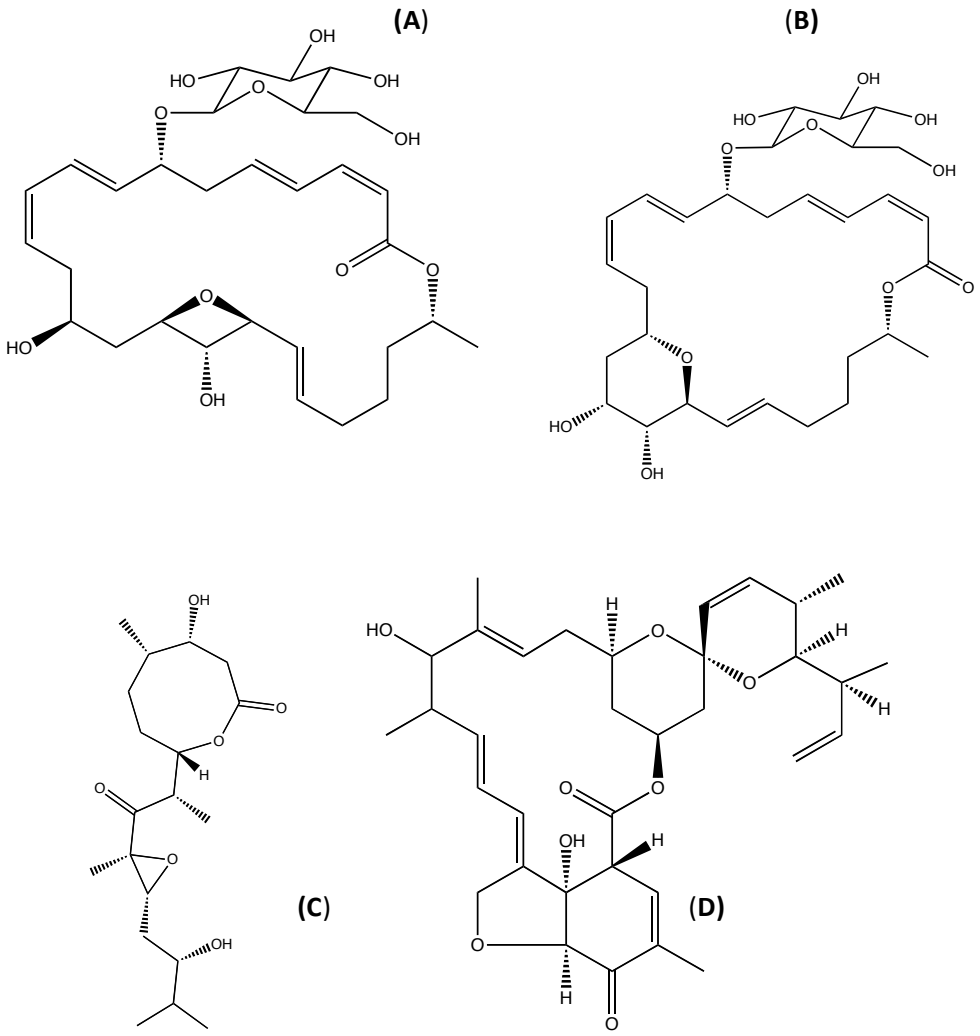


Figure 6-20 Chemical structures of (A) Glycosylated macrolactin A1; Formula: $C_{30}H_{44}O_{11}$; Mass: 580.29, (B) Glycosylated macrolactin B1; Formula: $C_{30}H_{44}O_{11}$; Mass: 580.29, (C) Octalactin A; Formula: $C_{19}H_{32}O_6$; Mass: 356.22 and (D) Possible avermectin precursor derivative; Formula: $C_{34}H_{44}O_8$; Mass: 580.30.

6.4.8.2 BA27 Exiguobacterium marinum strain Tf-80

Only one mass *m/z* 552.47 ($M + H^+$) potentially fitted the criteria of a drug-like product of MW less than 550. Upon searching *MarinLit*, only two matches were identified both of which are analogues of manzamine A (Figure 6-21) and previously isolated from a marine sponge of the same class (Tsuda *et al*. 1996; Watanabe *et al*. 1998) and are now known to be products of bacteria(J.-F. Hu *et al*. 2003). Manzamine A was also used as a standard in the HPLC-MS/MS network (section 5.4.3). If one of these were the compound responsible for activity, a match may be expected in the molecular network.

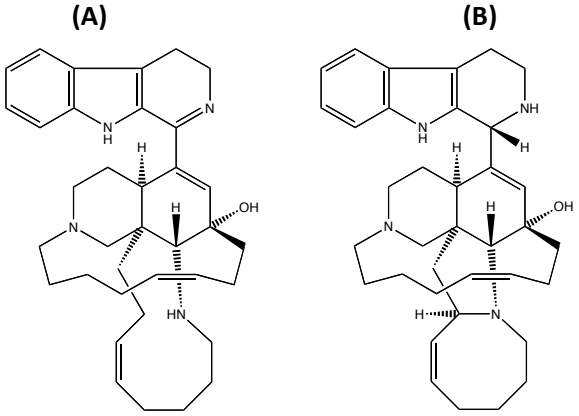


Figure 6-21 Chemical structures of (A) 3,4-Dihydromanzamine J; Formula: $C_{36}H_{48}N_4O$; Mass: 552.38 and (B) Manzamine D; Formula: $C_{36}H_{48}N_4O$; Mass: 552.38.

6.4.8.3 Novel antibacterial compounds

In total, 10 predicted masses did not match the mass of any of the expected ionisation species and were therefore considered novel. Four of these masses appeared in the common ionisation form of $(M + H)^+$ but unfortunately the masses identified were greater than 550 MW and were therefore not considered drug-like by this research project. The mass found from BA11.2 (*m/z* 326.4) had no matches upon further limitations and therefore was considered potentially novel and drug-like (MW < 500). However, more likely compounds were identified by taxonomically limiting by family instead of class, resulting in fewer matched compounds. With this altered limitation, only three of the twelve observed mass values displayed any matches (*m/z* 378.2, 580.3 and 356.3 for compounds BA11.2.1, BA11.3.1, BA11.3.3, respectively; (Table 6-8) when compared to likely ion masses using MarinLit, therefore identifying another 27 potentially novel ion masses and nine novel parent masses.

Table 6-8 Results of *MarinLit* search using the predicted *m/z* = $(M + H)^+$ with a taxonomy limitation of 'family' for both the parent sponge (*H. panicea*) and bacteria (BA11; *Streptomyces violascens* strain G8A-22 and BA27; Exiguobacterium marinum strain Tf-80).

Sample ID	Source	Compound name	Molecular formula	Exact Mass	Reference
BA11.2 (1)	Bacteria	R-indomycinone	$C_{22}H_{18}O_6$	378.11030	(Schumacher *et al*. 1995)
	Bacteria	Actinofuranone A	$C_{22}H_{34}O_5$	378.24060	(Cho *et al*. 2006)
	Sponge	Cholest-4,6,8(14),22E-tetraen-3-one	$C_{27}H_{38}O$	378.29220	(Ciminiello *et al*. 1989)
Ba11.3 (1)	Bacteria	Octalactin A	$C_{19}H_{32}O_6$	356.21990	(Tapiolas *et al*. 1991)
	Sponge	Panicein-C	$C_{21}H_{24}O_5$	356.16240	(Cimino *et al*. 1973)
Ba11.3 (2)	Bacteria	Glycosylated macrolactin A1	$C_{30}H_{44}O_{11}$	580.2884	(Mondol and Shin 2014)
		Glycosylated macrolactin B1			

In summary, searching by parent mass with two potential taxonomic sources (bacteria and sponge) gave a large number of identification options in *MarinLit*. Consequently, potential leads may have been dismissed easily without definitive identification. This method was suitable for sponge samples but too many results have been returned for the bacterial samples, reducing the number of leads. A large number of the active bacterial spots also had higher molecular weights, therefore displaying a greater degree of complexity, where this is a potential limitation if the activity was explored further in terms of ease of identification and the eventual pharmaceutical properties. Many of the identities of compounds could be resolved by the use of HRMS, which may be beneficial in completing a complete chemical summary of compounds isolated. However, exploring 'known compounds' further does not fulfil the principal aim of this thesis of attempting to isolate novel active molecules.

One limitation of searching the marine specific *MarinLit* database was that it only identified products extracted directly from sponges, therefore excluding identification of any precursors or semi synthetic analogues that have not been previously extracted directly from sponges. However, this does not discount the effectiveness of the methods employed as analogues that have never been isolated previously may be identified. This process essentially bio-mines nature instead of producing synthetic analogues in a laboratory.

6.4.9 Molecular networking as a dereplication strategy for antibacterial natural products from bacterial extracts.

Following analysis of the network (Figure 6-22), any direct matches between the standards and the samples were considered identification of that standard compound within that

sample. No direct matches were found; therefore confirming the bacteria cultivated are not producing the compounds represented by the standards. Any circumstance where a standard was directly linked within a network to a sample (via an edge) it was concluded that structurally similar compounds were found in that sample. The clusters 1, 3, 6 and 16 contained a mix of standards and sample and were analysed further.

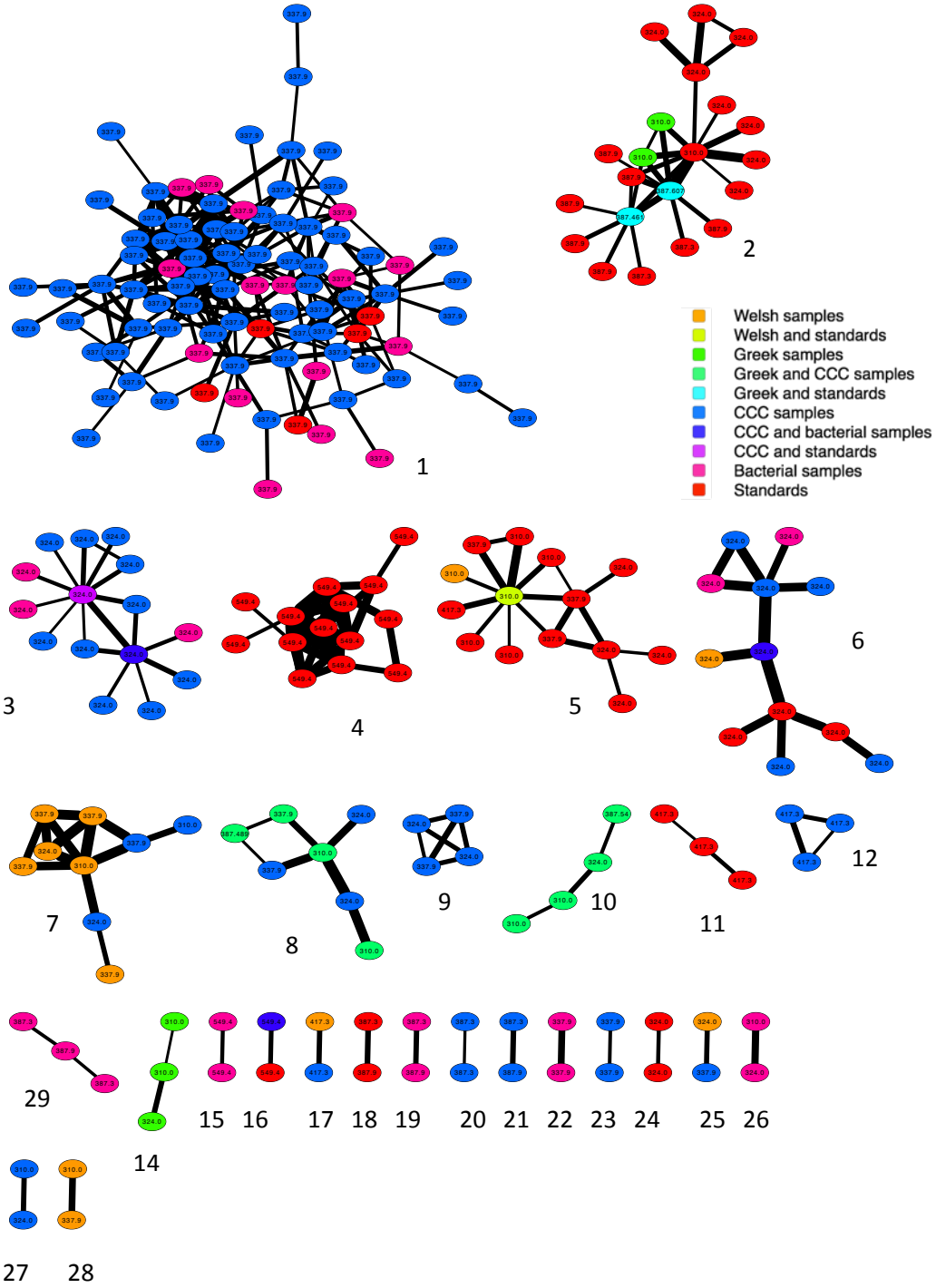
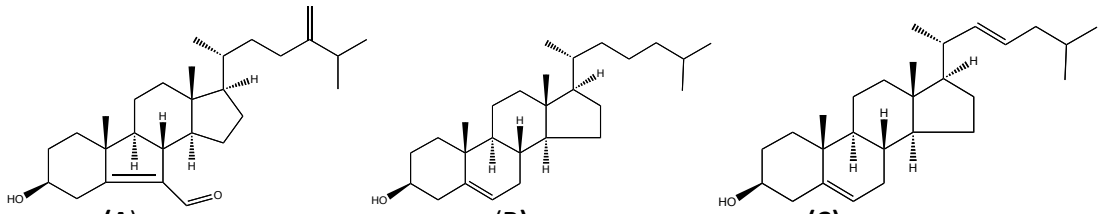


Figure 6-22 Molecular network of HPLC-MS/MS data. Each node represents a parent mass with an individual fragmentation pattern. The node may represent fragmentation patterns from more than one sample (if deemed the same). Each edge represents the cosine similarity between each node. The thicker the edge, the more closely related the compounds within the nodes are.

6.4.9.1 Analysis of the molecular network

Comparison was first made to the analysis completed in section 5.4.3 where cluster 1, 3, and 6 (13 in section 5.4.3) had already been analysed prior to the addition of the bacterial samples. Cluster 1 showed potential contamination of cholesterol, hence the appearance in further samples. Cluster 3 also appeared to show a match for cholesterol but upon analysis of the matched peaks, they were not well resolved and therefore did not confirm identification. All spectra found in Cluster 6 (13 in section 5.4.3) displayed a single distinct peak at *m/z* 186.23, which matched the standards oroidin, manoalide and cholesterol. This mass was found in multiple samples at the same Rf and was considered to be a contaminant in section 5.4.3.

However similar sterols have been isolated previously such as cholesterol directly from sponge (section 3.4.4) (B, Figure 6-23), the potential isolation of steroids such as that in Figure 6-17 and parguesterol A from BA11.4 (A, Figure 6-23) and the identification of sterols in a similar study (Viegelmann *et al*. 2014) (C, Figure 6-23). With such regular occurrence in other samples it is possible that similar complex terpenoids found in bacteria have been identified, rather than the run of cholesterol contaminating further samples.



(A) **(B)** **(C)**

Figure 6-23 Chemical structures of (A) parguesterol A (Ba11.4); Formula: $C_{28}H_{44}O_2$; Mass: 412.33, (B) cholesterol; Formula: $C_{27}H_{46}O$; Mass: 386.35 and (C) (3B)-Cholesta-5, 22-dien-3-ol; Formula: $C_{27}H_{44}O$; Mass: 384.34.

One hit that did not occur was that of BA27 and manzamine A, which may have occurred if the parent mass of BA27.1 represented a manzamine derivative (Figure 6-21). This is one of the limitations of this experiment as there was peak-picking of only the parent masses of standards and therefore if manzamine A was not present in the sample, no match would occur. In summary, the network failed to match any spectra from the bacterial samples to those of the active standards, suggesting that bacteria alone do not produce these exact compounds.

Future experiments could integrate the bioautography TLC-MS data into the network, therefore increasing the population of nodes making more direct matches likely and potentially linking active compounds to structurally similar standards. For example, manzamine D and another analogue (Figure 6-21) were the only potential compounds identified by TLC-MS but no matches were found in the current network as this parent mass was not searched. Completion of MS/MS on this mass and insertion of this data into the network would confirm the identity of this product if its fragmentation pattern was deemed similar to the standard manzamine A. Another interesting addition would be to test known standards from a particular species of sponge or bacteria, a good example of this is Panicein-C, which was potentially identified as an active compound isolated from the bacteria in this study. This could help confirmation of structure if the molecular network matched their fragmentation patterns. Rather than re-completing HPLC-MS/MS, this could also be done by fragmenting the products directly from a TLC plate using a different MS unit.

In summary no direct chemical links were identified between sponge and bacteria using TLC-MS or HPLC-MS/MS. An explanation for this could be that marine sponges supply a precursor or an environment that the bacteria require to produce the active product. This could be determined by incorporating sponge material directly into agar, which is common practice in some laboratories and essential for growing some sponge specific bacteria (Schmidt *et al*. 2000). Whether this addition would mimic the actual environment found on a sponge is however not guaranteed as the replicated environment on an agar is known not to be ideal as only 1 % of sponge bacteria is reported to be culturable. It was decided that it would not be accurate to calculate a similar figure from the information gained from this project using the complete microbiome 16S rRNA data and the isolated active species 16S rRNA data. This is due to the random nature of the colonies removal from plates and the identification of only active strains cultured.

6.5 Conclusion

In this chapter, a resource of marine sponges in an unusual and unexplored environment was investigated. Sponge associated populations of bacteria showed activity against clinically relevant organism and may present unique biosynthetic conditions that catalyses the biosynthesis of novel compounds.

The objective of growing uncommon bacteria producing antibacterial natural products was achieved by the use of low nutrient and selective saltwater agars. Integration of antibacterial and antifungal agents within the agar prevented the growth of faster growing more common bacteria. Combination of these parameters formed selective agars, which enhanced the growth of antibacterial strains of *Actinomycete* and *Streptomyces* species; targeted as known producers of novel active compounds prior to cultivation. The use of 16S rRNA analysis enabled the identification of the entire microbiome of bacteria present on the sponge prior to cultivation, which allowed direct comparison between species and any bacteria that were sponge species specific. The results showed that in the groups tested, *Spirochaetes* were species specific within samples, only appearing in detectable numbers in *H. perleve* sponge species or *H. panicea* found in direct competition with *H. perleve* sponge species.

Antibacterial activity was confirmed against clinically relevant bacteria MRSA and *E. coli,* thus indicating that potentially clinically relevant compounds were actively produced by the bacterial strains. Identification of the microbial species responsible for activity was completed using 16S rRNA analysis, which identified that four previously uncultured bacteria have been successfully cultured. This confirmed the methods developed to target novel bacteria were effective and increased the chance of finding a novel compound as the discovery rate of novel compounds is usually increased in taxonomically novel strains (Abdelmohsen *et al*. 2010).

Scaled-up culturing proved successful for the two species of bacteria tested, neither of which have been previously chemically profiled. Upon extraction with solvents, antibacterial activity was retained and identified in the acetone extracts. This was confirmed using the microdilution and bioautographic TLC methods. Dereplication of the compounds responsible for activity was completed by coupling the bioautographic data with TLC-MS. This method identified some potential leads, but was limited by searching for molecular weights of two taxonomic classes (bacteria and sponge) leading to an increase in database results. In future,

more structure specific information should be applied for dereplication, such as orthogonal spectroscopic techniques (*e.g.* NMR) or higher resolution mass spectrometry, to provide fragmentation data and accurate masses. It may be possible to fit either of these techniques directly in line with the current method of TLC-MS.

Completion of HPLC-MS/MS on bacterial extracts permitted direct integration of data into the molecular network formed from the products of marine sponge presented in section 5.4.3. This method identified only potential analogues of cholesterol in samples but showed no matches to known active compounds. This experiment was limited however by only comparing with the parent masses of standards. The next step in this project would be to complete a similar study to that previously reported but refine by searching for the masses identified as active products identified using TLC-MS. No direct chemical matches were reported between the marine sponge host and bacteria, although Panicein-C, which has only previously been directly isolated from the host sponge (Cimino *et al*. 1973) was potentially isolated from the cultured bacteria.

Chapter 7
General Discussion

7 General discussion

7.1 Discussion

Marine sponges are a well documented source of a diverse range of novel antimicrobial compounds and because of this, they have been extensively researched over the past 40 years (Laport *et al*. 2009; Rifai *et al*. 2005; Ankisetty and Slattery 2012; Forenza *et al*. 1971). The chemical characterisation of many sponges had been reported prior to the commencement of this PhD, but there was still considerable opportunity to isolate novel sources of activity within species that had not been explored. There was no scientific literature covering Welsh sponges as a source of novel compounds and therefore, the Welsh coastline provided a unique opportunity to exploit unstudied marine sponges and their medicinal potential.

It was previously recognised that a variety of sponges could be found in Welsh waters (Goodwin and Picton 2011) and established that the bacteria found on UK sponges show growth inhibitory activity against multiple strains of bacteria (Kennedy *et al*. 2009; Flemer *et al*. 2012). This research project collected sponge samples in a multitude of different environments including at depth via SCUBA and in the shallows of Severn estuary, which has the second largest tidal range in the world making it a truly unique environment. Collecting samples in different environments increases the chance of isolating a range of compounds and compounds in differing concentrations (Sacristán-Soriano *et al*. 2011).

Samples were collected from the Isle of Samos (Greece) and Pembrokeshire (Wales, UK), targeting unstudied species, in both environments, after completing a literature search. In total, 39 samples were collected from Greece and 30 from Wales. Nine different species were sampled from Greece including two species that have not been previously researched from a chemical perspective. Known species were also targeted for the varying conditions they were found in and as controls to validate the methods employed. Six different species were collected from Wales, all of which were specifically targeted for their lack of chemical characterisation.

One of the prime objectives of this thesis was to complete a comprehensive investigation of the antibacterial biological activity within a sponge from non-polar to polar compounds. A sequential extraction process was utilised, starting with non-polar extraction and progressing

to polar extraction. When investigating the chemical constituents of marine sponges, most other researchers used methanol or a mix of methanol and dichloromethane (Galeano *et al*. 2011; Ankisetty and Slattery 2012; Houssen and Jaspars 2012) for their primary extraction and if no non-polar solvents are used many non-polar compounds will not be extracted. This is disadvantageous because non-polar compounds will be instantly lost as they will not be extracted. The methods employed by this project proved successful, producing more than double the yield of similar research studies (Youssef *et al*. 2013; Kochanowska *et al*. 2008). Post-extraction of the collected samples in this project, a significant difference ($p < 0.05$) was recorded between the chemical composition of the sponges from Greece and Wales, with the majority of the Greek material being extracted in the non polar solvent (hexane) and the majority of the Welsh material being extracted in the polar solvent (methanol). This is tentative evidence that different growth environments do result in unique natural product synthesis with potentially more fats developing in the warmer Greek waters. This data is useful for future collection, enabling prediction of how much initial sponge material is needed to recover a desired quantity of any active material. It is also interesting as most other studies only completed their preliminary extraction with relatively polar solvent systems, therefore would not have extracted the majority of material found in the hexane extract of Greek sponges in this project.

To the best of this researchers knowledge, up to the commencement and the completion of this project, direct TLC-MS has never been used in tandem with bioautographic techniques in marine sponge antimicrobial research, although a similar method has been recently employed in a study of propolis (Kasote *et al*. 2015). Combination of these techniques allowed localisation of activity and effective visual comparison of activity differences between various sponge samples. Sponge extracts were tested against the clinically relevant bacteria MSSA, MRSA and *E. coli* (Johani *et al*. 2010; Davies *et al*. 2013) and the bioautographic technique identified activity in a large proportion of the extracts studied. A variation in retardation factor (Rf) of activity spots visible on a thin-layer chromatography (TLC) plate indicated that the antimicrobial activities within these samples were provided by different chemical entities (Kasote *et al*. 2015). Utilising mass spectrometry (MS) in tandem with bioautography, allowed rapid identification of antibacterial compounds from crude marine sponge extracts. Dereplication of known compounds was completed against a marine specific natural product database *MarinLit*, which proved an effective method of limiting compound hits, reducing them by over 85% in comparison to *SciFinder*, a comprehensive, but non-specific database. Using molecular weight as the search term, this study identified

42 predicted masses as *possibly novel* and 50 predicted masses were considered to be novel. Assembling this data together revealed 15 parent masses which were considered potentially novel (no likely matches for any possible ions) including Mf16 (*Crambe crambe*), 2w20 (*Dysidea fragilis)*, 2w14 (*Halichondria panicea*), 2u2, 2u3 (*Geodia sp*.) and Ms13 (*Aplysina aerophoba*). The majority of the molecular weights identified were also less than 550, defining them as drug-like by the stipulations set out in the project. Therefore, this project has successfully isolated novel drug-like leads from marine sponges.

HPCCC was employed as a method for extraction of pure compounds in greater yield than standard separation techniques such as HPLC and column chromatography. While HPCCC had previously been used for the isolation of sponge material (Jadulco 2002), no research has been published on the development of an activity based template solvent systems specific for the extracts produced from a sequential extraction method. The development of such a system, combined with the extraction method, permitted the isolation of active compounds with little or no loss of material. This was demonstrated by the isolation of one active compound in over 15-times the yield through HPCCC compared to column chromatography combined with thin-layer chromatography. The HPCCC method of separation of active compounds was validated by the appearance of some identical active parent masses following TLC-MS pre- and post-separation and the increased activity of the fractions separated via HPCCC to the crude extracts. The isolation of active samples with differing masses showed that Greek and temperate Welsh sponges are a rich source of diverse active metabolites and the ability to record retention time of each active fraction, the ease of adjustment of a four solvent based system and the direct scalability of HPCCC (DeAmicis *et al*. 2011) make this method perfect for future separations and targeting of specific active molecules.

While knowing the mass of any product could potentially prevent rediscovery, the disadvantage of this method was that without high-energy mass spectrometry to fragment the parent ion (Dunn *et al*. 2013) or completion of complementary spectroscopic techniques, such as NMR (Jaspars 1999), definitive identification of some compounds, including known compounds was not always possible. However, in this circumstance as unknown leads were found, definitive structural characterisation of known products was irrelevant as this was not the primary aim of this thesis.

The bioautographic technique was an excellent preliminary method of visualising activity within a crude extract and proved an effective method of narrowing leads when combined

with TLC-MS. However the antibacterial results were not quantifiable, thus other techniques were implemented to quantify the activity of an extract. The two most commonly used laboratory techniques for studying the antibacterial activity are the disc diffusion method and the microdilution method (Hughes and Fenical 2010), both of which were employed in this project. These quantifiable tests were completed for all extracts with 29% showing activity against MRSA and 28% showing activity against *E. coli*, in some cases as or more effective than the standard hospital treatment for MRSA of Vancomycin (Hope *et al*. 2013). This is very encouraging for the efficacy of the compounds present as the crude extracts are not pure compounds. No significant difference ($p > 0.05$) was observed between the antibacterial activity of temperate Welsh sponges to those from the warmer waters of Greece, thus supporting Welsh sponges as a potential source of novel antibacterial compounds.

This project fingerprinted a large proportion of extracts, allowing direct comparison to known standards using HPLC-MS/MS to form a molecular network based on a compound's fragmentation pattern. The generation of a molecular network from this data allowed the removal of a lot of the time consuming tasks of directly comparing spectra and looking for similarities (Yang *et al*. 2013). This method has developed substantially in the past few years in the analysis of bacteria including those found on a sponge (M. C. Wilson *et al.* 2014) but has not been used to analyse sponge extracts directly. This study reported HPLC-MS/MS of parent masses of commercially available natural product standards, and thus the successful identification of oroidin, cholesterol derivatives, hymenidin and sceptrin. Interestingly, hymenidin and oroidin were only identified in the sponge, *Agelas oroides,* that had been well characterised, supporting the rationale for collection of this well characterised sponge.

The disadvantage of the MS method employed by peak picking the masses of standards meant that the network created was not actively looking for unknown compounds. Completing a random peak fragmentation would have produced a molecular fingerprint but would not have actively searched for antibacterial compounds, which was the primary objective of this project. The production of the network using the method employed would allow future insertion of the active masses obtained through TLC-MS into this network. These active masses could also be inserted into the network by using direct TLC-MS/MS or peak picking HPLC-MS/MS data, thus achieving direct dereplication against standards and gathering more structural information. Previously it had been thought that accurate MS directly from a TLC plate was impossible, as this does not provide the controlled

environment of HPLC. However MS technology has improved rapidly over the past five years and direct accurate MS and fragmentation is now being reliably performed in incredibly varying environments, including the use of mini-MS in the field on backpacks (Hendricks *et al*. 2014).

Future work would ideally optimise each of the successful techniques reported in this thesis. Firstly, with bioautographic-TLC and TLC-MS, parent masses of interest could be mined using varying MS techniques coupled to the TLC-interface. HPLC-MS/MS could be incorporated to compare the fragmentation of these parent masses to standards as a method of dereplication, using a molecular network, and to potentially give an idea of the structural class of the active molecule. If a structure is still not fully characterised, HPCCC followed by MS/MS and NMR could be employed to separate the compound with little of no loss of material and then identified using more powerful spectroscopic techniques (Figure 7-1).

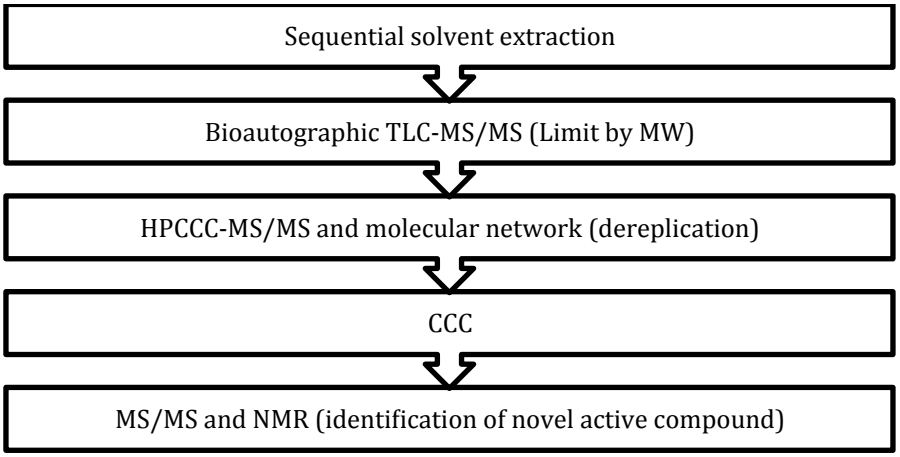


Figure 7-1 Suggested flow chart for future work exploring sponges as a source of antimicrobials.0p

A significant number of reports had previously isolated and identified microbes with antibacterial activity isolated from marine sponges collected in UK waters (Margassery *et al.* 2012; Kennedy *et al*. 2009; Santos *et al*. 2010; Baker *et al*. 2009). There was however no published research on UK sponges comparing the active metabolites extracted from these isolated bacteria to the parent sponge material. This PhD has uniquely achieved this by the combination of bacterial isolation and activity testing, with genetic identification techniques (16S rRNA analysis) and chemical profiling using the bioautographic overlay method combined with TLC-MS and HPLC-MS/MS. The study specifically targeted slow growing bacteria using low nutrient and specific marine agars to target *Actinomycete* and *Streptomyces* strains, which are known producers of novel active metabolites (Cho *et al*.

2006; Magarvey *et al*. 2004; Subramani and Aalbersberg 2012; Kwon *et al*. 2006; Kennedy *et al*. 2009). From the 'single colonies' isolated, 18% displayed activity against MRSA and 18 active single colonies were identified using 16S gene sequencing, four of which have not been previously cultured. This shows that not only has this project managed to culture some bacteria that had only ever been identified from a complete microbiome, these cultures can now be used in a future project for chemical profiling as the discovery rate of novel compounds is increased in taxonomically novel strains (Abdelmohsen *et al*. 2010).

Large-scale production of two strains of bacteria, *Streptomyces violascens* strain G8A-22 and *Exiguobacterium marinum* strain Tf-80, active against MRSA and E. coli, was also completed. Both of these tested strains were isolated from *Halichondria panicea* and neither had been previously chemically profiled. The bioautographic assay confirmed antibacterial activity for both strains with differences in Rf indicating different compounds were responsible for activity. *Streptomyces violascens* strain G8A-22 and *Exiguobacterium marinum* strain Tf-80 also displayed MICs of 1.28 mg mL^{-1} and 2.56 mg mL^{-1}, respectively against MRSA making them comparable to some of the whole sponge extracts extracted from *Halichondria panicea*. It must be noted however that some sponge crude samples also displayed significantly greater activity towards MRSA comparable to Vancomycin and this was not the case for the bacterial extracts. Bioautographic coupled TLC-MS matched no active compounds from the sponge extracts with those isolated from active bacteria. This shows that either these bacteria were not producing the compounds extracted from the sponge or when growing in isolation (without sponge), the bacteria were unable to produce these compounds. TLC-MS identified twelve masses that had no matches in literature. Unfortunately when limiting taxonomically by order, only one mass was identified as drug-like. When limiting by family, which is a more likely match, only three of the twelve observed mass values had any matches, leaving nine potentially novel parent masses.

Completing a molecular network using HPLC-MS/MS on marine derived bacteria has been shown previously (Yang *et al*. 2013; Winnikoff *et al*. 2013; Yue *et al*. 2012) but integrating the technique with the parent marine sponge data has not been previously attempted. Similar chemical profiling using the HPLC data has recently been described on UK marine sponge-microbe association, but not for the strains involved in this study or using the fragmentation pattern as the source of comparison (Viegelmann *et al*. 2014). When integrated into the network, no definitive links were identified with active standards although similar sterol-like substances were identified. Interestingly, these substances were also identified in another

UK study comparing the chemical profile of a bacteria isolated from a sponge to the parent sponge sample (Viegelmann *et al*. 2014) using a different profiling technique. The lack of crossover of active compounds did not necessarily proved that the associated bacteria were not the producers of active compounds within the sponge as there were a few limitations to this study. Firstly, looking at the whole sponge microbiome, it is clear that the bacteria isolated and tested only made up a very small proportion of all the bacteria present on the sponge. This suggests that the bacteria producing the active compounds on the sponge may not have been isolated and tested. It is also possible that the microbe required the presence of the sponge to produce these natural products. This could be resolved by incorporating sponge material into the agar, and had been used to prove sponge-microbe specificity in the past (Schmidt *et al*. 2000). Following on from this project, a similar study could be performed to determine the masses identified as antibacterial using TLC-MS/MS and integration into the formed molecular network in a method similar to that suggested for sponge extracts. More masses would then appear in the network, which if similar to commercial standards, would be identified in the molecular network.

The use of 16S rRNA analysis enabled the description of the entire microbiome of bacteria present on the sponge prior to cultivation, which allowed direct comparison between species and any bacteria that were sponge species specific. This is a commonly reported experiment and is now standard in sponge-microbe affiliation testing (Kennedy *et al*. 2009; Flemer *et al*. 2012; Viegelmann *et al*. 2014). The results showed some interesting specific interactions between microbe and host including *Spirochaetes*, which showed species specificity within samples, only appearing in visible numbers in *Hymeniacidon perleve* or *Halichondria panicea* found in direct competition with *Hymeniacidon perleve*. While species specificity of bacteria is not a novel concept, this particular relationship described has never been previously identified.

7.2 Conclusion

In summary, this project has demonstrated that multiple unstudied sponges, from unique locations, displayed activity against bacteria of clinical relevance in the UK. The source of this activity was isolated to novel parent masses, implying the presence of novel natural products, using a combination of techniques that has not been previously reported for the chemical profiling of marine sponges. In combination with mining the sponge directly for compounds, bacterial strains isolated from the sponge, including four previously uncultured

strains of microbe, displayed growth inhibitory activity against clinically relevant bacteria. These could be cultivated on a larger scale to allow further novel compounds to be isolated. Unfortunately, no structure matches occurred between the antibacterial sponge metabolites and the active bacterial metabolites. However this highlights that sponges alone, or in close bacterial symbiosis, are still good sources of novel metabolites compared to bacteria growing in isolation.

In conclusion, while marine sponges have already been successfully mined for novel natural products, this resource is not exhausted. Numerous opportunities still exist for finding novel antimicrobial compounds by exploiting innovation in technology, methods and natural evolution, to discover new leads to combat the increasing prevalence of antibiotic resistance.

Appendices

Appendix I

Archipelagos
Institute of Marine Conservation
www.archipelago.gr

4/10/2012

License for material transfer

To Whom It May Concern:

It is herby certified that Archipelagos Institute of Marine Conservation, hereby represented by its Manager & Head Scientist, Anastasia Miliou, agrees to provide Cardiff University's School of Pharmacy and Pharmaceutical Sciences, represented by Doctor Alex White, with biological substances and materials for research purposes, as is described in the enclosed ***Material Transfer Agreement.***

The biological substance in question comprises from marine sponges collected from the coastline surrounding the islands of Samos and Fourni, in the eastern Aegean Sea, Greece. The material currently transferred is 51 samples of 14 sponge species. All of the sponge samples are small and biologically inert, therefore dead, having been dried upon collection and stored in individual, sealed bags. This eliminates the potential for contamination and transport of such contaminants. The Sponges will be used in the field of pharmaceutical research by Cardiff University's School of Pharmacy and Pharmaceutical Sciences, following the terms described in the enclosed ***Material Transfer Agreement.***

ΑΡΧΙΠΕΛΑΓΟΣ
ΙΝΣΤΙΤΟΥΤΟ ΘΑΛΑΣΣΙΑΣ ΚΑΙ
ΠΕΡΙΒΑΛΛΟΝΤΙΚΗΣ ΕΡΕΥΝΑΣ ΑΙΓΑΙΟΥ
ΑΦΜ: 099634083 - ΔΟΥ: ΣΑΜΟΥ
ΠΟΤΑΜΙ ΜΕΣΟΚΑΜΠΟΥ - ΜΥΤΙΛΗΝΟΙ ΣΑΜΟΥ Τ.Κ. 83101
ΤΗΛ. 2273061191
info@archipelago.gr www.archipelago.gr

Anastasia Miliou

Manager & Head Scientist

Archipelagos, Institute of Marine Conservation

info@archipelago.gr

Marine Research Base
P.O. Box 42, Pythagorio,
83103, Samos, Greece
Tel: +30 22730 37533
Fax: +30 22730 37533

Centre of Environmental & Agronomic Research
P.O. Box 1, Rahes,
83301, Ikaria, Greece
Tel: +30 22750 41070
Fax: +30 22750 41673

Administrative Office
Mihail Voda 89,
10440, Athens, Greece
Tel: +30 210 8253024
Fax: +30 210 8253783

School of Earth and Ocean Sciences
Head of School and Distinguished Research Professor in Geomicrobiology R J Parkes
Ysgol Gwyddorau'r Ddaear a'r Môr
Pennaeth yr Ysgol ac Athro Ymchwil o Fri mewn Geomicrobioleg R J Parkes

CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

Cardiff University
Main Building
Park Place
Cardiff CF10 3AT
Wales UK
Telephone +44(0)29 2087 4630
Fax Direct +44(0)29 2087 1326
www.earth.cf.ac.uk
Prifysgol Caerdydd
Prif Adeilad
Plas y Parc
Caerdydd CF10 3AT
Cymru Y Deyrnas Gyfunol

24th September 2012.

To whom it may concern,

This is to confirm that Ms Helena long is working for Cardiff University and assisting in a sponge research programme. As part of this work, Ms Long will be transporting inert, dried and dead sponge material in small quantities for analysis in the UK. This is under the agreement of the research institute, Archipelagos, represented by Ms Anastasia Miliou (Head Biologist) who are supplying the material and Dr Alex White, Cardiff University School of Pharmacy and myself, Dr Rupert Perkins, Cardiff University School of Earth and Ocean Sciences, as supervisor of Ms Long's work in Greece. Transport of this material is under agreement covered by the UK department of the Environment and Rural Affairs (DEFRA) and fully meets the criteria allowing the transport of the said sponge material.

If there are any problems with the safe transit of the sponge materials transported by Ms Long, please contact me (24 h number) on 0044 77251 756 929.

Yours faithfully,

Dr Rupert Perkins
Director of Marine Geography
Head of marine and Coastal environments Research group,
School of Earth and Ocean Sciences,
Cardiff University.

School of Earth and
Ocean Sciences

Cardiff University

animalhealth
International Trade Centre for Imports
Government Buildings, Beeches Road, Chelmsford, Essex, CM1 2RU
Tele: 01245 464860, Fax: 01245 351162

TO WHOM IT MAY CONCERN

Date: 19th November 2010

Importation of animal products derived from Reptiles, Amphibians, Fish, Invertebrates or Honey from other EU member States

The Importation of Animal Products and Poultry Products Order 1980 (as amended) under which we issue licences for imports of certain animal derived material for research purposes from EU countries, does not cover products derived from (reptiles/amphibians/fish/invertebrates/honey). There are no animal health concerns in respect of products derived from (reptiles/amphibians/fish/invertebrates/honey) and as such you will not need an animal health import licence from Animal Health or Defra. There are restrictions and prohibitions on the carriage of certain products and substances by post, guidance for which is available from the following sites:
http://www.dft.gov.uk/pgr/freightdgt1/
http://www.liv.ac.uk/biomedsci/safety/infectious_substances.ppt
http://www.postoffice.co.uk/portal/po/content1?catId=19100182&mediaId=19100263&print=true
http://www.royalmail.com/portal/rm/content1?catId=400138&mediaId=36200679

I would suggest you include a copy of this letter with any consignments of this material you bring in from EU countries in case you experience any problems on entry.

Please note this letter only covers products, if you wish to import live animals of any of the species mentioned, you will need to contact;
AHITchelmsford@animalhealth.gsi.gov.uk

Yours sincerely

DEPARTMENT FOR ENVIRONMENT, FOOD AND RURAL AFFAIRS

Specialist Service Centre for Imports

Head Office: Animal Health Corporate Centre, Block C, Government Buildings, Whittington Road, Worcester WR5 2LQ
t +44(0)1905 763355 f +44(0)1905 768851 e corporate.centre@animalhealth.gsi.gov.uk
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www.defra.gov.uk/animalhealth

Welsh School of Pharmacy
Head of School Professor Stephen Denyer
Ysgol Fferylliaeth Cymru
Pennaeth yr Ysgol Yr Athro Stephen Denyer

CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

Cardiff University
Redwood Building
King Edward VII Avenue
Cardiff
Wales
UK
Tel Ffôn
Fax Ffacs
www.
Prifysgol Caerdydd

26th July 2012

To Whom It May Concern:

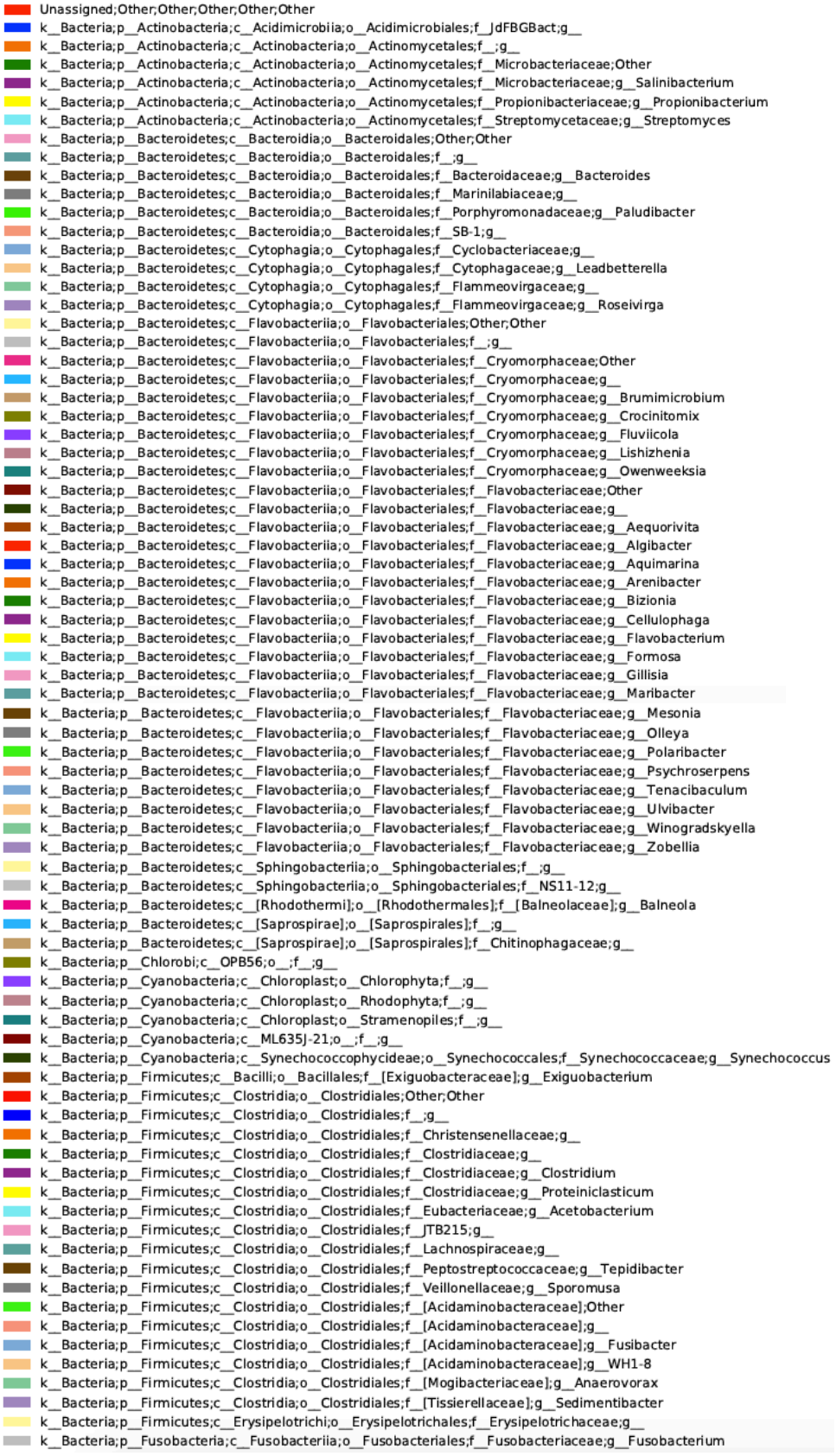
I am an academic at Cardiff University in the United Kingdom with a research interest in the medical potential of marine natural products. We have engaged Helena Long, a Cardiff University student studying at Archipelagos in July/August 2012, to collect some small samples of local sponges. These organisms have been proven to produce molecules with interesting medical potential.

Based on survey data supplied to us by Archipelagos, we have identified a number of sponge species that are readily available in the local area. Small samples will be collected, dried and returned to Cardiff to screen for anti-cancer and anti-microbial activity.

Information gained from screening (i.e. any positive 'hits' that are identified) will be used to justify support for further research. Archipelagos would be part of these discussions as an important collaborator.

Dr Alex White

Appendix II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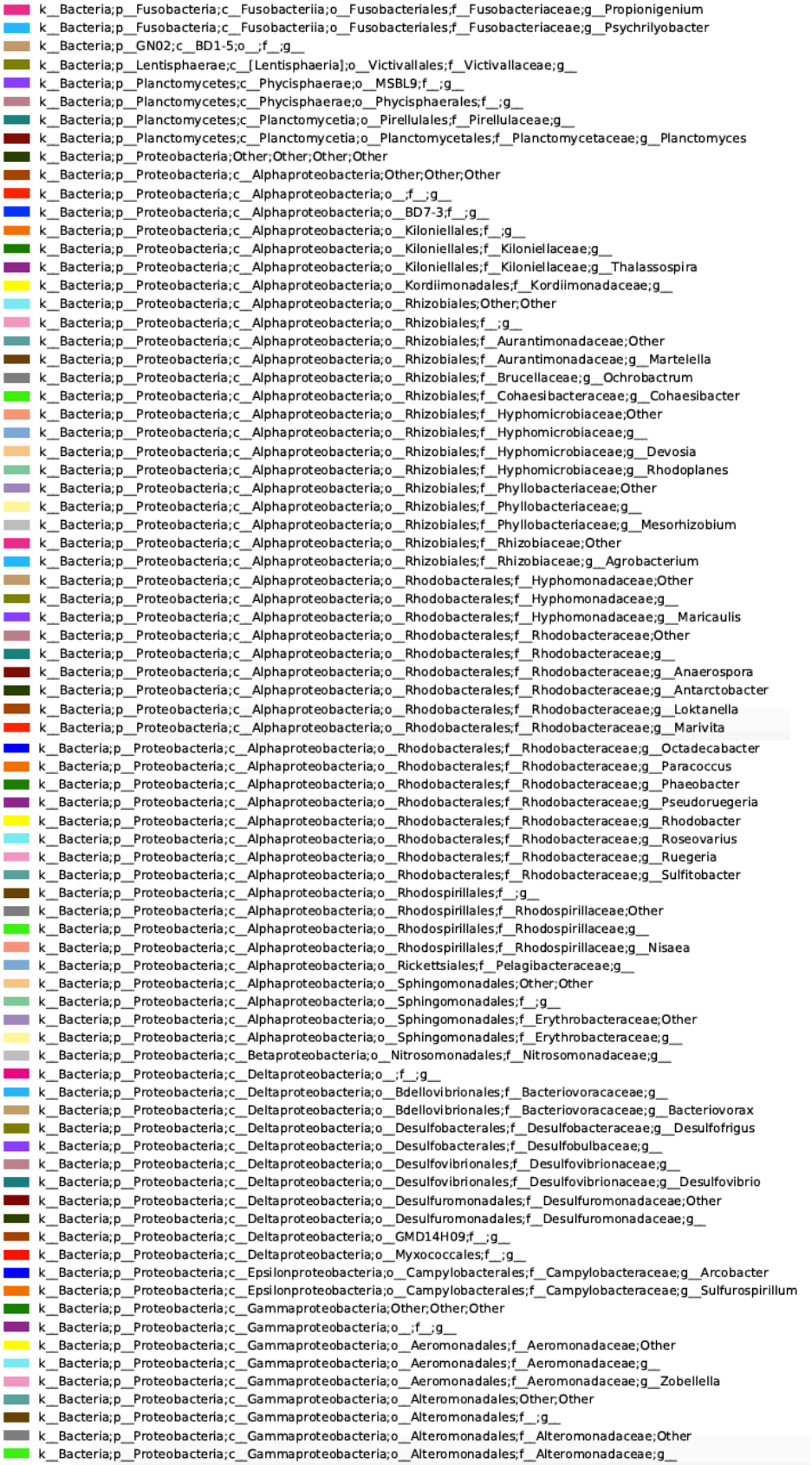
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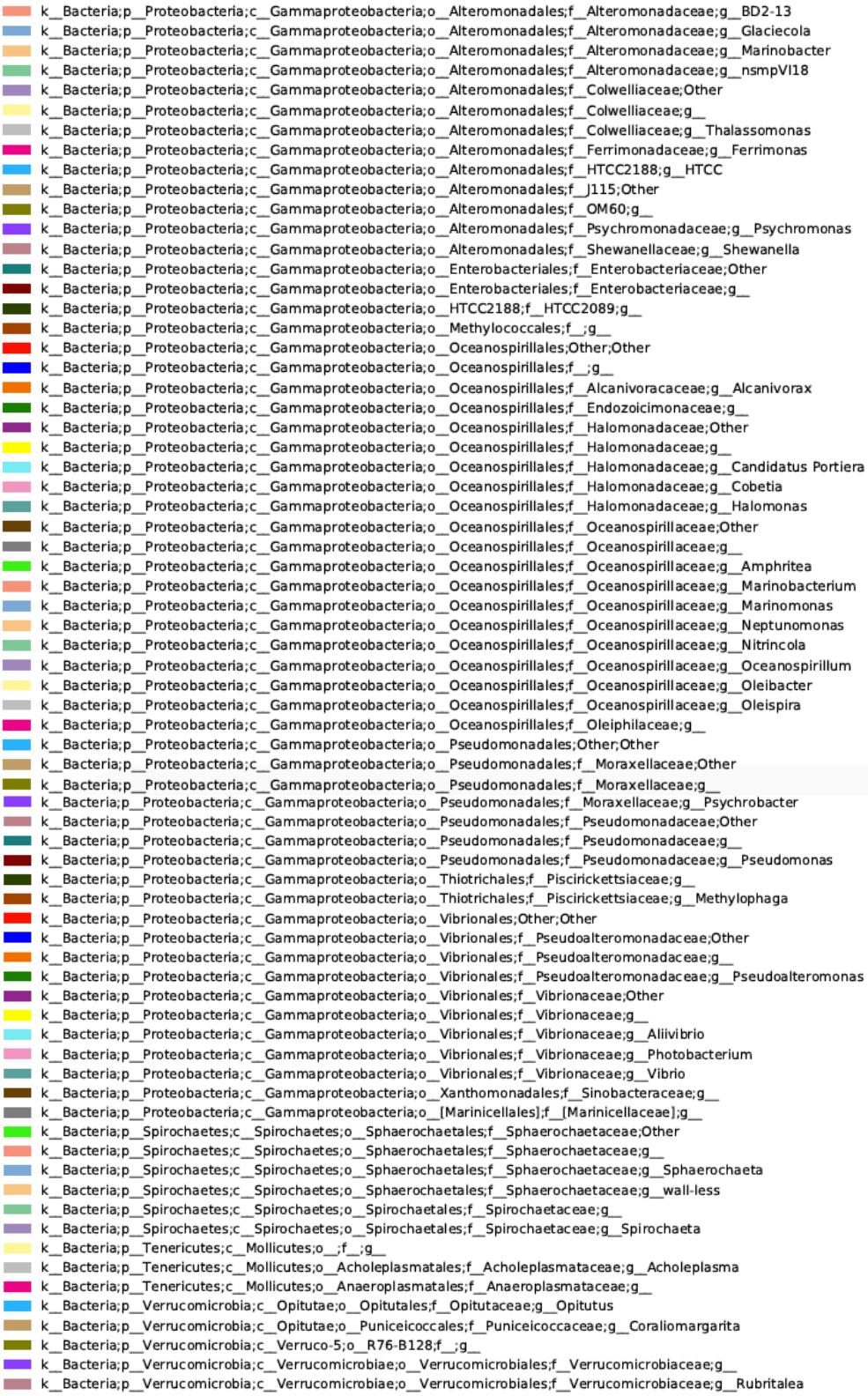
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k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oceanospirillaceae;g__Oceanospirillum
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oceanospirillaceae;g__Oleibacter
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oceanospirillaceae;g__Oleispira
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oleiphilaceae;g__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;Other;Other
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;Other
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Psychrobacter
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;Other
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Thiotrichales;f__Piscirickettsiaceae;g__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Thiotrichales;f__Piscirickettsiaceae;g__Methylophaga
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;Other;Other
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Pseudoalteromonadaceae;Other
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Pseudoalteromonadaceae;g__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Pseudoalteromonadaceae;g__Pseudoalteromonas
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;Other
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Aliivibrio
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Photobacterium
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Vibrio
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacteraceae;g__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__[Marinicellales];f__[Marinicellaceae];g__
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Sphaerochaetales;f__Sphaerochaetaceae;Other
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Sphaerochaetales;f__Sphaerochaetaceae;g__
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Sphaerochaetales;f__Sphaerochaetaceae;g__Sphaerochaeta
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Sphaerochaetales;f__Sphaerochaetaceae;g__wall-less
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Spirochaeta
k__Bacteria;p__Tenericutes;c__Mollicutes;o__;f__;g__
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Acholeplasmatales;f__Acholeplasmataceae;g__Acholeplasma
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales;f__Anaeroplasmataceae;g__
k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__Opitutales;f__Opitutaceae;g__Opitutus
k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__Puniceicoccales;f__Puniceicoccaceae;g__Coraliomargarita
k__Bacteria;p__Verrucomicrobia;c__Verruco-5;o__R76-B128;f__;g__
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__Rubritalea

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